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Mgr. Zdeněk Musil

Molekulárně biologická analýza feochromocytomu a paragangliomu

Molecular biological analysis of pheochromocytoma and paraganglioma

Disertační práce

Školitel: RNDr. Kateřina Hirschfeldová, Ph.D.

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Obsah:

1. Seznam použitých zkratek:

1.LF UK a VFN	1. lékařská fakulta Univerzity Karlovy a Všeobecné fakultní nemocnice
2.LF UK a FN Motol	2. lékařská fakulta Univerzity Karlovy a Fakultní nemocnice v Motole
18F-FDA	[18F]-fluorodopamin
^{18}F-FDG	[18F]-fludeoxyglukóza
^{18}F-FDOPA	[18F]-fluoro-dihydroxyphenylalaninem
^{131}I-MIBG	metaiodobenzylguanidin
aCGH	array CGH
AD	autosomálně dominantní
ATRX	ATP-dependent helicase X-linked
BRAF	serine/threonine-protein kinase, protooncogene
CDH1	cadherin-1
CDKN2A	cyclin-dependent kinase 2A
CDKN2B	cyclin-dependent kinase 2B
CGH	comparative genome hybridization, komparativní genová hybridizace
DGGE	denaturační gradientová gelová elektroforéza
DNA	deoxyribonukleová kyselina
EDTA	ethylendiamintetraoctová kyselina
FAD	flavinadenindinukleotid
FH	fumarate hydratase
FHIT	fragile histidine triad
FGFR1	fibroblast growth factor receptor 1
FMTC	familiální medulární karcinom štítné žlázy
FN	fakultní nemocnice
GIST	gastrointestinálními stromálními nádory
GNAS	guanine nucleotide binding protein, alpha stimulating
H_2O	voda
HCl	chlorovodíková kyselina
HIF	hypoxia-induced factor
HIF2A (EPAS1)	hypoxia-inducible factor 2 Alpha, endothelial PAS domain protein
HNPGLs	head and neck paragangliomas, paragangliomy hlavy a krku
H-RAS	HRas protooncogene
IDH1	isocitrate dehydrogenase 1

IDH2	isocitrate dehydrogenase 2
KIF1Bß	kinesin family member 1Bß
KMT2D	lysine methyltransferase 2D
K-RAS	Kirsten rat sarcoma viral oncogene homolog
LM PCR	ligation mediated PCR
MAX	MYC associated factor X
MAML3	mastermind like transcriptional coactivator 3
MDH2	malate dehydrogenase 2
MEN 2	syndrom mnohočetné endokrinní neoplázie typu 2
MET	MET protooncogene, receptor tyrosine kinase
min.	minuta
MTC	medular thyroid carcinoma, medulární karcinom štítné žlázy
mTOR	mammalian target of rapamycin
NaOH	hydroxid sodný
NF1	neurofibromin 1
NGFR	nerve growth factor receptor
nM	nanomol
N-RAS	NRAS proto-oncogene, GTPase
PARP	poly (ADP-ribose) polymerase
PCR	polymerase chain reaction, polymerázová řetězová reakce
PET	pozitronová emisní tomografie
PET/CT	pozitronová emisní a RTG počítačová tomografie
PGL	paragangliom
PGL1	syndrom paragangliomu 1
PGL2	syndrom paragangliomu 2
PGL3	syndrom paragangliomu 3
PGL4	syndrom paragangliomu 4
PGL5	syndrom paragangliomu 5
PHEO	feochromocytom
PHEO/PGL	feochromocytom/paragangliom
PHD2/EGLN1	egl-9 family hypoxia inducible factor 1
pM	picomol
µl	mikrolitr
RET	rearranged during transfection, protooncogen

SeqCap	sequence capture
SDH	sukcinát dehydrogenáza
SDHA	sukcinát dehydrogenáza, podjednotka A
SDHAF2	succinate dehydrogenase complex assembly factor 2
SDHB	sukcinát dehydrogenáza, podjednotka B
SDHC	sukcinát dehydrogenáza, podjednotka C
SDHD	sukcinát dehydrogenáza, podjednotka D
TE pufr	tris/EDTA pufr
TERT	telomerase reverse transcriptase
TMEM 127	transmembrane protein 127
TP53	tumor protein p53
TRIS	trisaminomethan
VHL	von Hippel-Lindau tumor suppressor
WHO	World Health Organization, Světová zdravotnická organizace
Wnt	Wingless/Int-1 signální dráha

2. Abstrakt:

Předkládaná práce shrnuje výsledky výzkumu, který se zabývá relativně vzácnými neuroendokrinními nádory - feochromocytomem a paragangliomem (PHEO/PGL).

Tyto nádory mohou vznikat díky dědičné genetické predispozici. Zpočátku své práce jsem se proto zaměřil na genetické vyšetření pacientů s PHEO/PGL. Byly zavedeny metody denaturační gradientové gelové elektroforézy, sekvenování dle Sangera, multiplex ligation probe amplification pro diagnostiku změn nejdříve v genech *SDHD, SDHB a RET*. Postupně se rozšiřoval (a stále i rozšiřuje) počet vyšetřovaných genů. V současné době metodou next generation sequencing vyšetřujeme následující geny: *ATRX, BRAF, CDH1, CDKN2A, CDKN2B, FGFR1, FH, FHIT, GNAS, HIF2A (EPAS1), H-RAS, IDH1, IDH2, KIF1Bß, KMT2D, K-RAS, MAML3, MAX, MDH2, MET, NF1, NGFR, N-RAS, PHD2/EGLN1, RET, SDHA, SDHAF2, SDHB, SDHC, SDHD, TERT, TMEM 127, TP53 a VHL.* Výskyt genových variant v uvedených genech (23%) se u českých pacientů s FEO/PGL se liší v porovnání s některými zahraničními studiemi (27%, 40%). Takovéto rozmezí může být způsobeno geografickými rozdíly nebo výběrem pacientů.

PHEO/PGL se většinou (75%) vyskytují v benigní formě. Pro maligní formu onemocnění svědčí výskyt chromafinní tkáně v místech, kde se obvykle nenalézá, jako jsou např. játra, plíce či kosti. V testovaném souboru pacientů jsme se zaměřili na hledání dalších znaků, které by mohly svědčit pro maligní průběh, např.nižší věk pacientů s prvním výskytem nádoru, větší velikost tumoru a sekrece norepinefrinů.

Pro maligní formy PHEO/PGL zatím neexistuje účinná léčba. Mezi současné možnosti léčby patří např. chirurgický zákrok, použití chemoterapie pomocí kombinace cyclofosfamidu, vincristinu a dacarbazinu, podávání terapeutických dávek ^{131}I- metaiodobenzylguanidinu, nebo použití prostředků cílené molekulární terapie (např.inhibitory angiogeneze, hypoxia inducible faktoru, histon deacetylázy aj.). V další fázi práce jsme zkoušeli hledat další cílené léčebné možnosti. V souboru nasbíraných nádorových tkání, jsme testovali přítomnost mutace genu *BRAF* a případné terapeutické použití BRAF inhibitorů.

Klíčová slova: feochromocytom, paragangliom, next generation sequencing, gen SDHB, gen *BRAF*

3. Abstract:

This work summarizes the results of a research inquiring into relatively rare neuroendocrine tumors – pheochromocytomas and paragangliomas (PHEO/PGL)

These tumors may arise on a hereditary genetic predisposition basis. On that account we primarily focused on a genetic examination of patients with PHEO/PGL. Methods for diagnostics of changes in *SDHD, SDHB and RET* genes were implemented. The number of examined genes has been (and is still being) extended. Currently we are investigating these genes: *ATRX, BRAF, CDH1, CDKN2A, CDKN2B, FGFR1, FH, FHIT, GNAS, HIF2A (EPAS1), H-RAS, IDH1, IDH2, KIF1Bβ, KMT2D, K-RAS, MAML3, MAX, MDH2, MET, NF1, NGFR, N-RAS, PHD2/EGLN1, RET, SDHA, SDHAF2, SDHB, SDHC, SDHD, TERT, TMEM 127, TP53 and VHL*, using next generation sequencing. The number of variations of the above mentioned genes is different (23%) in Czech patients with PHEO/PGL in comparison with some foreign studies (27%, 40%). This may be caused by geographical influences or selection of patients.

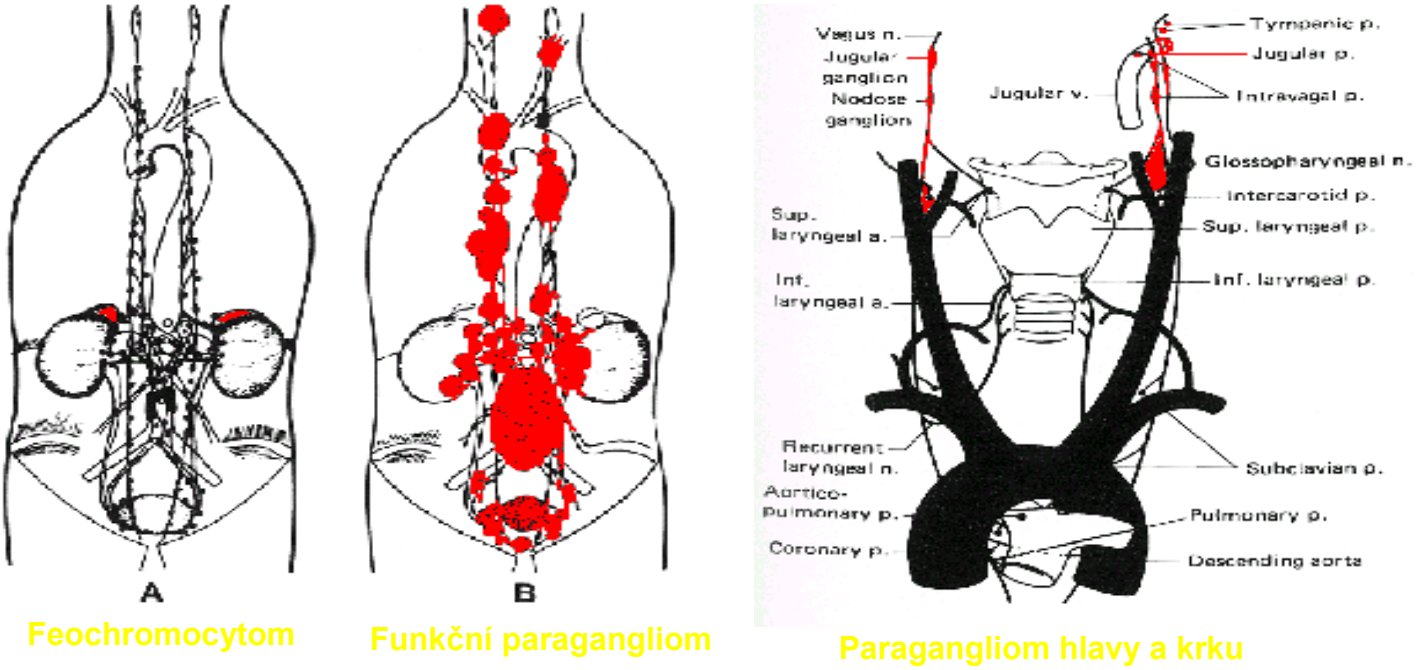
PHEO/PGL occur mainly (75%) in a benign form. A malignant form may be indicated by the presence of chromaffin tissue in locations where these tumors do not usually occur – liver, lungs, bones. In our study we focused on characteristics indicating the malignancy, for example, the lower age of patients with the first manifestation of PHEO/PGL, larger tumor size or norepinephrine secretion.

There is no effective treatment for malignant forms of PHEO/PGL. Treatment possibilities include surgical resection of the tumor, chemotherapy with cyclophosphamide, vincistrine and dacarbazine, therapeutic ^{131}I - metaiodobenzylguanidine doses, or targeted molecular therapy (inhibitors of angiogenesis, hypoxia-inducible factors, histone deacetylases). We are trying to find new treatment possibilities. In our tissue samples we tested the presence of the *BRAF* mutations and possible therapeutic application of BRAF inhibitors.

Key words: pheochromocytoma, paraganglioma, next generation sequencing, *SDHB* gene, *BRAF* gene

4. Úvod:

Feochromocytomy (PHEO) a paragangliomy (PGL) jsou relativně vzácné nádory, které vznikají v chromafinní tkáni. Podle světové zdravotnické organizace (WHO) pod pojmem PHEO se označují nádory, které jsou lokalizované ve dřeni nadledvin, oproti tomu PGL se nachází extraadrenálně a dále se rozdělují na krční (HNPGLs) a břišní (Obr. 1).



Obr. 1: Místa výskytu feochromocytomů a paragangliomů (Lips et al. 2006)

(HNPGLs) mají původ v parasympatické tkáni, většinou neprodukují katecholaminy (hormonálně "němé") a jsou rozpoznávány v důsledku útlaku okolních struktur. Břišní paragangliomy vychází ze sympatické tkáně, mohou se vyskytovat od baze lební až po pánevní dno, velmi často se nachází v místě odstupu arteria mesenterica inferior tzv. Zuckerkandlově orgánu (Ronald et al. 2004). Mezi širokou odbornou veřejností jsou tyto nádory známy jako *nádory 10%* (bilaterální výskyt, maligní, geneticky podmíněné, extraadrenální, atd.), avšak výsledky studií provedených v nedávné době ukazují až na přítomnost kauzálních mutací u přibližně 30–40 % výskyt kauzálních mutací. (Boedeker C.C. et al. 2011, Karásek D. et al. 2010, Bausch B. et al. 2012, Neumann H.P. et al.2002).

5. Diagnostika PHEO/PGL:

Výskyt těchto druhů onemocnění je doprovázen řadou příznaků. Mezi nejčastější z celkem až 50 popisovaných příznaků patří hypertenze (trvalá i záchvatovitá), nauzea, pocení, palpitace. Nerozpoznání nebo chybná diagnóza však mohou být fatální. (Jafri M. et al. 2012, Widimsky J. Jr. Et al. 2009, Widimsky J. Jr. Et al. 2007, Zelinka T. et al. 2007).

5.1 Biochemická vyšetření u pacientů s PHEO/PGL

PHEO/PGL mohou metabolizovat, skladovat a vylučovat katecholaminy. Krátký výskyt katecholaminů v plazmě bohužel ztěžuje rozlišení patologické nadprodukce od normální zvýšené sekrece během stresu. Vzhledem ke výše zmíněným skutečnostem může měření katecholaminů poskytnout vysokou míru falešně pozitivních výsledků, zatímco odběr mezi záchvaty může způsobovat falešně negativní výsledky. Proto se doporučuje pro diagnostiku PHEO/PGL používat měření volných plazmatických metanefrinů nebo frakcionovaných močových metanefrinů (Lenders J.W.M. et al. 2014). Ačkoliv jsou volné plazmatické metanefriny a normetanefriny odstraňovány z oběhu téměř stejně rychle jako jejich katecholaminové prekurzory, jsou pro diagnostiku lepší než katecholaminy. Tyto metabolity jsou uvolňovány z katecholaminů kontinuálně a pomocí katechol-O-methyltransferázy přeměňovány na metanephrin a normetanephrin. Tento proces je nejen kontinuální, ale také nezávislý na exocytotické sekreci katecholaminů, která může být u PHEO/PGL přerušovaná nebo jen velmi málo aktivní (Eisenhofer G. et al. 2004; Eisenhofer G. Et al. 2012). Plazmatické frakcionované metanephriny mají vysokou senzitivitu (96–100%) a specificitu (85–89%), a proto je výhodné je použít při diagnostice rizikových pacientů, tj. např. pacientů s předchozí anamnézou PHEO, rezistentní hypertenzí, v rámci syndromologického onemocnění, s rodinnou anamnézou genetického syndromu nebo adrenálním incidentalomem naznačujícím přítomnost PHEO. Kromě toho mohou být plazmatická měření užitečná u dětí, u kterých je obtížné odebírat moč 24 hodin (Lenders et al. 2014). Další metodou volby může být 24 hodinový sběr frakcionovaných močových metanephrinů. Tato metoda vykazuje vysokou senzitivitu (97%) a specificitu (91%) (Perry et al. 2007).

5.2 Zobrazovací metody u pacientů s PHEO/PGL

U pacientů s biochemicky potvrzeným PHEO/PGL jsou používány zobrazovací metody pro zjištění lokalizace primárního tumoru, případně metastáz. Počítačová tomografie (CT) a magnetická rezonance (MRI) jsou vysoce senzitivní metody a umožňují přesný popis a vymezení nádoru. U většiny pacientů je anatomické zobrazení doplněno funkčními zobrazovacími metodami, které definitivně PHEO/PGL potvrdí. Mezi nejvíce používané funkční metody patří scintigrafie s [^{123}I]-metaiodobenzylguanidinem (MIBG) a pozitronová emisní tomografie (PET) s radiofarmaky,jako např. 6-[18F]-fluorodopaminem (18F-FDA) a [18F]-fluoro-dihydroxyphenylalaninem (18F-FDOPA) (Hoegerle S. et al. 2002; Pacak K. et al. 2001; Timmers H.J. et al. 2009). U maligních forem PHEO/PGL způsobených zejména mutacemi genu sukcinát dehydrogenázy, podjednotky B (*SDHB)* jsou metastázy lépe zobrazeny pomocí ^{18}F-fluorodeoxyglukózové PET v kombinaci s CT (^{18}F-FDG PET/CT) (Timmers H.J. et al. 2007; Taeib D. et al. 2009). Zvýšené vychytávání ^{18}F-FDG může být považováno za známku změněného geneticky podmíněného glukózového metabolismu buňky (Favier J. et al. 2009).

6. Genetika PHEO/PGL:

Jak PHEO tak PGL se mohou vyskytovat v rámci genetických syndromů, do současné doby bylo popsána celá řada takovýchto syndromologických onemocnění (Qi X.P. et al. 2012, Björklund P. et al. 2016, Butz J.J. et al. 2017).

6.1 Syndromy spojené s PHEO/PGL

6.1.1 Mutace sukcinát dehydrogenázy (SDH)

6.1.1.1 Syndrom paragangliomu 1 (PGL1); gen *SDHD* (OMIM 602690)

Nádory syndromu PGL1 jsou většinou benigní nádory HNPGLs, riziko výskytu PHEO/PGL se pohybuje mezi 16–21 % (Boedeker C. et al 2014). HNPGLs jsou většinou biochemicky němé (Benn D.E. et al. 2006), ve 20 % však produkují dopamin nebo jeho metabolit methoxytyramin, což může být využito při monitorování pacientů (van Duinen N. et al. 2013). Mutace genu sukcinát dehydrogenázy, podjednotky D *(SDHD)* vede téměř výhradně k rozvoji onemocnění, je-li zděděna od otce (parent of origin efect) (Baysal B.E. et al. 2011, Musil et al. 2012). Penetrance u nosičů *SDHD* mutací se pohybuje mezi 40–100 % (Hensen E.F. et al. 2011). PHEO se v rámci PGL1 syndromu vyskytují v časnějším věku než HNPGLs (Boedeker C. et al. 2014). V rámci tohoto syndromu se mohou také vyskytovat nádory ledvin a žaludku (Favier et al. 2015).

6.1.1.2 Syndrom paragangliomu 2 (PGL2); gen *SDHAF2 (SDH5)* (OMIM 601650)

Gen *SDHAF2* je důležitý pro správnou inkorporaci flavinadenindinukleotid. kofaktoru (FAD) SDHA podjednotky sukcinátdehydrogenázy, která je nezbytná pro správnou funkci SDH komplexu (Opocher G. Et al. 2011). Mutace *SDHAF2* jsou vzácné a vedou téměř výhradně k rozvoji HNPGLs u jedinců ve věku 22–47 let. (Kunst HP et al. 2011).

6.1.1.3 Syndrom paragangliomu 3 (PGL 3); gen *SDHC* (OMIM 605373)

Nádory způsobené mutacemi genu *SDHC* jsou většinou solitární HNPGLs (vzácně se nacházejí i multifokálně a v maligní formě). Průměrný věk výskytu je 38 let (Jafri M. et al. 2012).

6.1.1.4 Syndrom paragangliomu 4 (PGL4); gen *SDHB* (OMIM 115310)

Tento syndrom je charakteristický výskytem paragangliomů v oblastech břicha, pánve a

mediastina (Neumann HP et al. 2004), v relativně mladém věku 25–30 let (Gimenez-Roqueplo A.P. et al. 2012). Mutace *SDHB* vedou často k rozvoji maligní formy onemocnění.

6.1.1.5 Syndrom paragangliomu 5 (PGL5); gen *SDHA* (OMIM 614165)

Zárodečné mutace genu *SDHA* se nacházejí u pacientů s PHEO/PGL stejně jako u pacientů s gastrointestinálními stromálními nádory (Burnichon N et al. 2010, Pantaleo M.A. et al. 2011). Bialelické mutace byly detekovány u pacientů Leighovým syndrome (Horvath R. et al. 2006).

6.1.2 Syndrom mnohočetné endokrinní neoplázie typu 2 (MEN 2); gen *RET* (OMIM 171400)

Syndrom MEN 2 je autosomálně dominantně (AD) dědičné onemocnění způsobené mutacemi v genu rearranged during ransfection *(RET).* V rámci syndromu MEN 2 se vyskytují medulární karcinomy štítné žlázy (medular thyroid carcinoma MTC), PHEO a hypertyreóza (Lebeault M. et al. 2017). MEN 2 se dále rozděluje na tři podtypy: MEN 2A, MEN 2B a familiální MTC (FMTC). Protoonkogen *RET* kóduje protein kinázový receptor, který skládá se z 21 exonů. 90 % případů syndromu MEN 2 je způsobeno bodovými mutacemi v exonech 10, 11, 13, 14 a 15 (Qi X.P. et al. 2012, Plaza-Menacho I. et al. 2018). PHEO/PGL se nachází přibližně u 50% pacientů s MEN 2 (Castinetti F. et al. 2017). PHEO se může nacházet v obou nadledvinách, maligní forma je ovšem poměrně vzácná (Wells S.A. et al. 2018).

6.1.3 Syndrom von Hippel - Lindau; gen *VHL* (OMIM 193300)

Syndrom von Hippel - Lindau je AD dědičné onemocnění s výskytem mutací v genu von Hippel-Lindau *(VHL)*. Klinicky se toto syndromologické onemocnění projevuje hemangioblastomy retiny a centrálního nervového systému, světlobuněčným karcinomem ledvin, PHEO a mnohočetnými cystami ledvin a pankreatu (Bouhamdani N. et al. 2017, Plevova P. et al. 2009).

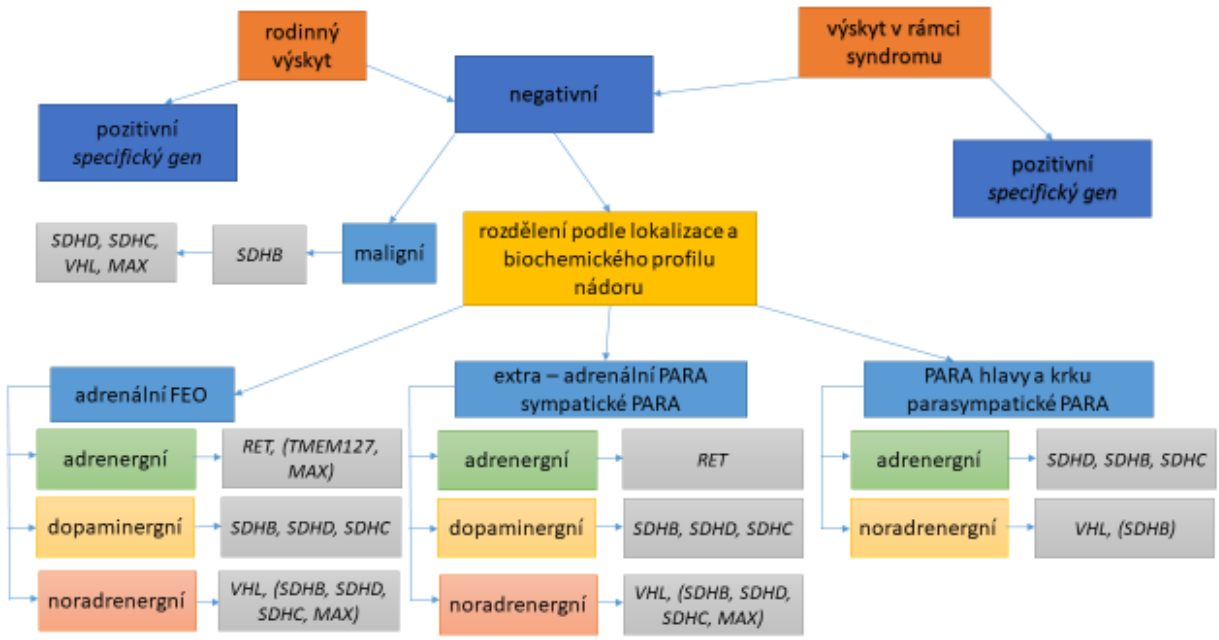
6.1.4 Syndrom neurofibromatózy typu 1 - NF1; gen *NF1* (OMIM 162200)

Neurofibromatóza typu 1 (též morbus von Recklinghausen nebo periferní typ neurofibromatózy) je AD onemocnění způsobené mutacemi genu neurofibromin 1 (*NF1).* Mezi klinické projevy syndromu NF1 patří tzv. „skvrny barvy bílé kávy, café-au-lait spots“, Lischovy noduly, neurofibromy, pihy v oblasti axil či třísel, gliomy optiku, PHEO (Radtke H.B. et al. 2007, Pillai S. et al. 2016).

Mutace v dalších genech jako např. *MAX, TMEM127, EPAS1/HIF2A, FH, EGLN1* a *KIF1* se u pacientů s PHEO/PGL vyskytují méně častěji (Mercado-Asis L.B. et al. 2018, Welander J. et al. 2018).

6.2 Genetické vyšetření pacientů s PHEO/PGL

V současné době vyšetřujeme metodou next generation sequencing (NGS) následující geny: *ATRX, BRAF, CDH1, CDKN2A, CDKN2B, FGFR1, FH, FHIT, GNAS, HIF2A (EPAS1), H-RAS, IDH1, IDH2, KIF1Bβ, KMT2D, K-RAS, MAML3, MAX, MDH2, MET, NF1, NGFR, N-RAS, PHD2/EGLN1, RET, SDHA, SDHAF2, SDHB, SDHC, SDHD, TERT, TMEM 127, TP53 a VHL.* Není-li zavedena metoda NGS může se použít diagnostika jednotlivých genů podle uvedeného schématu (Obr. 2).



Obr. 2: Algoritmus genetického vyšetření u pacientů s PHEO/PGL (upraveno dle Musil et al. 2012, Lenders et al. 2014)

Tab. 1: Přehledná charakteristika PHEO/PGL: DO - dopamin, E – epinefrin, NE – norepinefrin, NMN – normetanefrin, MN – metanefrin, MT - metoxytyramin (upraveno dle Musil et al. 2012)

Gen	**Lokus**	**Riziko malignity**	**Primární výskyt**	**Biochemie**
SDHA	5p15	neznámé	bez predilekce	neznámé
SDHB	1p 36.13	31 – 71%	extra adrenálně	DO nebo MT, MN, NMN
SDHC	1q 21	nízké	paragangliomy hlavy a krku	MNM, MN, DO nebo MT, žádné
SDHD	11q 23	<5%	paragangliomy hlavy a krku (multifokální výskyt)	MNM, MN, DO nebo MT, žádné
SDHAF2 (SDH5)	11q 13.1	nízké	paragangliomy hlavy a krku (multifokální výskyt)	neznámé
VHL	3p 25 - 26	5%	adrenálně	NMN, NE
NF1	17q 11.2	12%	adrenálně	MN, MNM
RET	10q 11.2	<5%	adrenálně (bilaterálně)	E, MN
MAX	14q23.3	střední	adrenálně (bilaterálně)	neznámé
TMEM127	2q11.2	nízké	adrenálně	MN

6.3 Genetické klastry PHEO/PGL

Různé molekulárně biologické, epigenetické a genomické studie rozdělily na základě výskytu mutací, methylačního a transkripčního profilu PHEO/PGL do několika klastrů.

V klastru 1 jsou zahrnuty mutace v genech důležitých pro detekci množství kyslíku nebo geny kódující enzymy Krebsova cyklu jako např. *SDHx, VHL, FH, HIF2A* nebo *EGLN1/2.* V klastru 2 jsou zařazeny geny podílející se na kinázové signalizaci, translaci genů a neurální diferenciaci *RET, NF1, TMEM127*, *MAX* a *KIF1Bβ.* Klastr 3 zahrnuje Wnt signální dráhu (Burnichon N. et al. 2011, Pang Y. et al. 2019).

7. Cíle práce:

Během svého postgraduálního studia jsem se spolupodílel na výzkumu neuroendokrinních nádorů PHEO/PGL. Ve své práci jsem se nesoustředil na jeden vědecký cíl, ale v průběhu výzkumu jsme řešili další vědecké otázky.

1. PHEO/PGL mohou vznikat na základě genetického podkladu. Jedním z prvních cílů bylo zavedení metod pro genetické testování pacientů a zjištění prevalence genetických změn u pacientů s PHEO/PGL.

2. Pro maligní formy těchto druhů nádorů svědčí přítomnost chromafinní tkáně v místech, kde se normálně nevyskytuje např. játra, kosti nebo plíce. Dalším cílem bylo nálézt doplňující znaky, které by mohly poukazovat na maligní průběh onemocnění.

3. Pro léčbu maligních forem PHEO/PGL se používá několik druhů léčby, nejúčinnější je zatím chirurgické odstranění nádorů, mezi další patří např. chemoterapie, podávání terapeutických dávek [^{131}I]-MIBG nebo cílená terapie dle molekulárního podkladu. V další fázi výzkumu jsme se zaměřili na hledání nových léčebných možností.

8. Materiál a metody:

8.1 Charakteristika souboru analyzovaného metodou next generation sequencing

Pro analýzu mutací metodou NGS jsme použili DNA pacientů z III. Interní kliniky 1.LF UK a VFN, Kliniky dětské hematologie a onkologie 2.LF UK a FN Motol a III. Interní kliniky FN Olomouc. Celkem jsme provedli analýzu 108 pacientů (56 žen a 58 mužů), ve věku od 19 do 85 let. U 83 pacientů (77 %) byl diagnostikován PHEO, u 25 pacientů (23 %) byl diagnostikován PGL (viz Tab. 2 Charakteristika analyzovaného souboru).

Tab. 2: Charakteristika analyzovaného souboru

pohlaví	věk	druh onemocnění	mutace
Ž	44	feochromocytom	neg.
Ž	66	paragangliom mnohočetný	SDHB c.689G>A, p.Arg230His
Ž	71	feochromocytom	neg.
M	57	feochromocytom	neg.
Ž	36	paragangliom abdominální	TMEM127 c.679A>G, p.Ile227Val
Ž	35	feochromocytom	TMEM127 c.159del, p.Trp53CysfsTer28
M	73	feochromocytom	neg.
Ž	61	feochromocytom	neg.
M	24	feochromocytom	neg.
Ž	41	feochromocytom	neg.
Ž	42	feochromocytom	MAX c.223C>T, p.Arg75*
Ž	37	feochromocytom	neg.
M	68	paragangliom abdominální	neg.
Ž	71	feochromocytom	neg.
M	57	feochromocytom	neg.
M	53	paragangliom abdominální	neg.
M	61	feochromocytom	neg.
Ž	40	feochromocytom bilaterální	RET c.1901G>T, p.Cys634Phe
Ž	49	feochromocytom	neg.
M	56	paragangliom abdominální	neg.
Ž	84	feochromocytom	neg.
Ž	27	feochromocytom bilaterální	neg.

pohlaví	věk	druh onemocnění	mutace
M	19	paragangliom abdominální	SDHB c.689G>A, p.Arg230His
M	41	feochromocytom	neg.
Ž	51	paragangliom mediastina	neg.
Ž	63	paragangliom abdominální	neg.
M	55	feochromocytom	neg.
Ž	62	feochromocytom	neg.
M	39	feochromocytom	neg.
M	59	feochromocytom	NF1 c.4547_4548insA, p.Asp1516_Lys1517fs
M	72	paragangliom mediastina	neg.
Ž	69	feochromocytom	neg.
Ž	31	feochromocytom	neg.
M	30	feochromocytom	neg.
M	67	feochromocytom	MET c.2962C>T, p.Arg988Cys
M	74	feochromocytom	neg.
M	67	paragangliom abdominální	SDHB c.689G>T, p.Arg230Leu
Ž	64	feochromocytom	neg.
Ž	55	feochromocytom	neg.
M	57	feochromocytom	neg.
M	84	feochromocytom	neg.
Ž	47	paragangliom abdominální	neg.
M	24	paragangliom mediastina	SDHC c.397C>T, p.Arg133Ter
M	27	paragangliom mnohočetný	SDHC c.397C>T, p.Arg133Ter
M	55	paragangliom mnohočetný	SDHC c.397C>T, p.Arg133Ter
M	46	feochromocytom	neg.
M	59	feochromocytom	MET c.3029C>T, p.Thr1010Ile
M	71	feochromocytom	neg.
Ž	73	paragangliom abdominální	neg.
Ž	59	feochromocytom	neg.
M	32	paragangliom abdominální	SDHD c.361C>T, p.Gln121Ter
Ž	75	feochromocytom	neg.
Ž	76	feochromocytom	neg.
M	48	feochromocytom bilaterální	neg.
M	48	feochromocytom	neg.

pohlaví	věk	druh onemocnění	mutace
Ž	46	feochromocytom	VHL c.481C>T, p.Arg161Ter
Ž	78	feochromocytom	neg.
Ž	74	feochromocytom	neg.
M	67	feochromocytom	neg.
M	47	feochromocytom	neg.
Ž	50	feochromocytom	neg.
Ž	85	feochromocytom	neg.
Ž	33	feochromocytom	neg.
M	27	feochromocytom bilaterální	VHL c.340+2T>C
M	38	paragangliom abdominální	SDHB c.689G>A, p.Arg230His
M	39	feochromocytom	neg.
Ž	74	feochromocytom	neg.
Ž	69	feochromocytom	neg.
Ž	50	paragangliom mnohočetný	neg.
M	60	feochromocytom	neg.
Ž	64	paragangliom abdominální	neg.
M	54	feochromocytom	neg.
Ž	66	feochromocytom	neg.
M	48	paragangliom mnohočetný	SDHB c.287G>A, p.Gly96Asp
M	50	feochromocytom	neg.
M	55	paragangliom abdominální	neg.
Ž	79	paragangliom malé pánve	neg.
M	55	feochromocytom	neg.
M	46	feochromocytom	neg.
M	43	feochromocytom	neg.
Ž	71	feochromocytom	neg.
Ž	74	feochromocytom	neg.
Ž	41	feochromocytom	neg.
Ž	70	feochromocytom	neg.
Ž	65	feochromocytom bilaterální	MAX c.97C>T, p.Arg33Ter
Ž	32	feochromocytom	neg.
M	39	paragangliom mnohočetný	SDHB c.137G>A, p.Arg46Gln
M	75	feochromocytom	neg.

pohlaví	věk	druh onemocnění	mutace
Ž	38	feochromocytom bilaterální	RET c.1900T>C, p.Cys634Arg
M	58	feochromocytom	neg.
Ž	49	feochromocytom	RET c.2372A>T, p.Tyr791Phe
Ž	56	feochromocytom	neg.
M	28	paragangliom mnohočetný	SDHC c.397C>T, p.Arg133Ter
Ž	78	feochromocytom	neg.
M	46	feochromocytom	neg.
Ž	74	feochromocytom	neg.
Ž	34	feochromocytom	RET c.833C>A, p.Thr278Asn
M	54	feochromocytom bilaterální	RET c.1900T>C, p.Cys634Arg
Ž	72	feochromocytom	neg.
M	61	feochromocytom	neg.
Ž	18	feochromocytom bilaterální	neg.
Ž	77	feochromocytom	neg.
M	74	feochromocytom	neg.
Ž	49	feochromocytom	neg.
M	83	feochromocytom	neg.
M	72	feochromocytom	neg.
Ž	81	paragangliom mnohočetný	neg.
Ž	64	feochromocytom	neg.

DNA byla izolována z periferní krve pacientů na automatické izolační lince QIAcube od firmy QIAGEN dle doporučeného izolačního protokolu.

Pro detekci změn na úrovni DNA používáme metodu NGS. Vzorky na sekvenaci jsou zpracovávány dle uvedeného postupu.

8.2 Postup přípravy vzorků na NGS

Sequence capture se sondami NimbleGen single Capture

Den 1.

1. **Fragmentace**

Změřit koncentraci DNA ve vzorku přístrojem nanodrop

Naředit DNA vzorek - 400 ng v TE pufru do celkového objemu 30 µl.

Z mrazáku vyndat: Condition solution, Kapa Fragmentation Buffer a Kapa Enzyme – KAPA Hyper Plus Library Preparation Kit.

Předchladit cycler na 4°C.

Na ledu připravit fragmentačního master mix:

Tab. 3: Fragmentační master mix

	1 vzorek	**12 vzorků** (10% rezerva)	**24 vzorků** (10% rezerva)
Condition solution	5 µl	66 µl	132 µl
Kapa Frag Buffer	5 µl	66 µl	132 µl
Kapa Enzyme	10 µl	132 µl	264 µl

Master mix po 20 µl přidat k vzokům DNA – pracovat na ledu.

Vzorky vložit do cycleru na program KAPA FR: 37°C – 10 min (teplota víka 47°C)

4°C – ∞

Po skončení uložit vzorky na led a pokračovat dalším krokem.

2. End-Repair & A-tailing

Rozmrazit A-tailing pufr a A-tailing enzym (fialová víčka) a připravit master mix:

Tab. 4: End-Repair a A-tailing master mix

	1 vzorek	**12 vzorků** (10% rezerva)	**24 vzorků** (10% rezerva)
A-Tailing Buffer	7 µl	92,4 µl	184,8 µl
A-Tailing Enzyme	3 µl	39,6 µl	79,2 µl

K vzorku (50 µl nafragmentované DNA) přidat 10 µl Master mixu.

Vzorky vložit do cycleru na program KAPA AT: 65°C – 30 min (teplota víka 85°C)

4°C – ∞

Vyndat adaptéry SeqCap Adapter Kit A/Kit B a nechat rozmrazit.

3. Ligace adaptérů

Při prvním otevření naředit adaptéry na zásobní koncentraci 10 µM: adaptér + 50 µl vody.

Rozmrazit ligation pufr, ligation enzym (žlutá víčka) (Kapa hyper plus kit) a PCR vodu.

Připravit ligační premix:

Tab. 5: Ligační premix

	1 vzorek	**12 vzorků** (10% rezerva)	**24 vzorků** (10% rezerva)
H_2O	5 µl	66 µl	132 µl
Lig pufr	30 µl	396 µl	792 µl
Ligasa	10 µl	132 µl	264 µl

Ke každému vzorku (60 µl produktu po úpravě konců) přidat:

- 5 µl adaptéru
- 45 µl ligačního premixu

Zapsat použitý adaptér

Vzorky vložit do cycleru a pustit program Ligace: 20°C – 15 min.

Vyndat z lednice AmPure XP Beds a vytemperovat 30 min při lab. teplotě.

4. Post-Ligation cleanup

Připravit čerstvý 80% ethanol.

Vytemperované AmPure Beads před použitím řádně zvortexovat.

Produkt po ligaci adaptérů (110 µl) převést do 1,5 ml eppendorfky.

Ke každému vzorku přidat 88 µl AmPure Beads.

1) Vortex
2) Inkubace 5 min (možno až 15 min pro nachytání většího množství DNA)
3) Magnet cca 30s - 2minuty
4) Po vyčeření roztoku odsát a odstranit supernatant
5) 2x opláchnout peletu: přidat 190 µl a odsát 200 µl čerstvého 80% etanolu
6) Po druhém propláchnutí odstranit všechen etanol
7) Vysušit peletu při lab. teplotě nepřesušit
8) Peletu resuspedovat 53 µl Tris HCl pH 8
9) Inkubace 2 min
10) Na magnet, cca 30s
11) Supernatant (50 µl) do čisté zkumavky, kuličky vyhodit

5. Ořez velikosti štěpených fragmentů na velikost 250-450bp pomocí AMPure XP reagent

První selekce (0,6x) odstraní fragmenty větší jak 450bp

1) K vzorku DNA (50 µl) přidat 35 µl AmPure XP Beads
2) Dobře vortexovat
3) Inkubace 5 minut (naváží se fragmenty delší než 450bp) + popis nových zkumavek

4) Připravit novou sadu zkumavek pro přenesený supernatant
5) Magnet 30 s (do vyčeření)
6) Přenést 80 µl supernatantu do nové zkumavky

Druhá selekce (0,8x) odstraní fragmenty menší než 250bp

1) K vzorku DNA (75 µl) přidat 10 µl AmPure XP Beads
2) Dobře vortexovat
3) Inkubace 5 minut (naváží se fragmenty delší než 250bp) + připravit 0,2 ml zkumavky na LM PCR
4) Magnet 30 s (do vyčeření)
5) Odsát a odstranit supernatant
6) 2x opláchnout peletu. Přidat 190 µl odebrat 200 µl čerstvého 80% etanolu
7) Po druhém propláchnutí odstranit všechen etanol (možno stočit na stolní centrifuze a znovu dat na magnet a odsát zbytek supernatantu)
8) Vysušit peletu při lab. teplotě (peleta ztmavne) nepřesušit
9) Peletu resuspedovat 23 µl 10mM Tris-HCl
10) Inkubace 2 min
11) Na magnet, cca 30s
12) Supernatant (20 µl) do čisté zkumavky.

6. Precapture LM-PCR

Z SeqCap Adapter kitu A rozmrazit zkumavku Pre LM-PCR oliga – navrchu LP1. Při prvním použití Pre LM-PCR oliga resuspendovat – stočit lyofilizovanou peletu, přidat 550 µl H_20.
Z Accessory kitu rozmrazit zkumavku se zeleným víčkem – Kappa hifi hot start ready mix (Kapa Hyper plus)- lepší rozalikvotovat á 25 µl.
Připravit Master mix:

Tab. 6: Precapture LM-PCR master mix

	1 vzorek	**12 vzorků** (5% rezerva)	**24 vzorků** (5% rezerva)
KAPA HiFi HotStart Ready Mix	25 µl	315 µl	630 µl
Pre-LM-PCE Oligos 1&2 (LP1)	5 µl	63 µl	126 µl

K 20 µl resuspendovaného vzorku přidat 30 µl Master mixu.

Umístit do cycleru a pustit na program LM1 PCR:

Tab. 7: Program LM1 PCR

Teplota		čas	Počet cyklů
98°C		45 s	1x
98°C		15 s	9 cyklů (8x opakovat)
60°C		30 s	
72°C		30 s	
72°C		60 s	1x
4°C		∞	

7. Post LM1 PCR Clean-Up

Amplifikované produkty již pokud možno uchovávat pouze v LOW-BINDING eppendorfkách.

1) Vzorky (50 µl) převést do Low-binding 1,5 ml eppendorfek a přidat 90 µl AmPure Beads.
2) Vortex
3) Inkubace 5 min
4) Magnet cca 30s
5) Odsát a odstranit supernatant
6) 2x opláchnout peletu 200 µl čerstvého 80% etanolu
7) Po druhém propláchnutí odsát supernatant, vyhodit, stočit kuličkami ven, odsát zbyteček etanolu
8) Vysušit peletu při lab. t. NEPŘESUŠIT! Nesmí popraskat! Dramaticky klesá výtěžek!
9) Peletu resuspedovat 53 µl PCR vody
10) Inkubace 2 min
11) Na magnet, cca 30s
12) Supernatant (50 µl) do čisté zkumavky

Přeměřit koncentraci na Nanodropu. Koncentrace by měla být více jak 1 µg a čistota 260/280 mezi 1,7-2,0 (±1,9). Koncentraci možno s větší přesností změřit na Qubitu.
Doporučeno analyzovat produkty na Agilentu DNA 1000 chip – produkty mezi 150-500 bp.

8. Alikvotování SeqCap sond SeqCap EZ Choice Library

Vyvarovat se opakovaným cyklům rozmražování a zmražování- při prvním použití

rozalikvotovat.

Do PCR zkumavek rozpipetovat á 2,25 µl.

9. Poolování knihovny

Do další reakce je třeba 1 µg fragmentů dohromady od všech vzorků (musí být proporcionální).

1 µg / počet vzorků = x (množství každého vzorku v µg) x . 1000 (převedení na nanogramy) / koncentrace z nanodropu (ng/µl) = µl každého vzorku

12 vzorků je 85µg DNA z každého vzorku=1µg

Napipetovat od každého vzorku vypočtený objem do 1,5 ml low-bind eppendorfky označené pool a datum.

10. Příprava hybridizační směsi s knihovnou

K poolu vzorků v 1,5 ml eppendrofce přidat: 5 µl Universal Blocking Oligos + 5 µl COT Human DNA a zvortexovat.

1) Do eppendorfky obsahující hybridizační směs s knihovnou přidat dvakrát větší objem AMPure Beads (1:2), homogenizovat protažením pipetou.
2) Inkubace 5 min RT
3) Dát na magnetický stojánek a po vyčeření (1min) odsát a zlikvidovat supernatant
4) Oplach 170 µl 80% EtOH (na magn. stojánku), čirý supernatant důkladně odsát
5) Nechat cca 15 min (max) schnout. Peletu nepřesušit!!
6) Rozmrazit z *EZ hybridisation and Wash kitu: Hybridisation Component A* (č. 6, zelené víčko) a *2x hybridisation buffer* (č. 5, hnědé víčko).
7) Připravit směs: 3 µl *Hybridisation Component A* (zelené víčko) + 7,5 µl 2x *hybridisation buffer* (hnědé víčko)
8) Vyndat hybridizační směs z magnetického stojánku a přidat 10,5 µl směs komponenty A s pufrem, inkubace 2 min RT
9) Dát na magnetický stojánek, nechat vyčeřit
10) Supernatant (10,5 µl) přepipetovat do nové 0,5 ml zkumavky
11) K vzorku přidat 4,5 µl SeqCap EZ probe pool
12) Vortex 10 sec

11.Hybridizace

Umístit do cycleru a pustit: 95°C 5 min

47°C 16 - 20 hod (víko na minimálně 57°C)

Den 2 .

Nahřát termoblok na 47°C.

Rozmrazit z EZ hybridisation and Wash kitu zkumavky 1, 2, 3, 4 (pufry mohou být vysrážené, po vytemperování se rozpustí) a 25x Bead wash buffer (lahvička 7).

Vždy nově naředit pufry:

Tab. 8: Příprava Wash pufrů

	Objem pufru:	PCR H_2O:	Celkový objem:	Teplota:
10x Stringent Wash buffer - **4**	40 µl	360 µl	400 µl	47°C
10x wash buffer I - **1**	30 µl	270 µl	300 µl	
10x wash buffer II - **2**	20 µl	180 µl	200 µl	
10x wash buffer III - **3**	20 µl	180 µl	200 µl	
2,5x Bead wash buffer - **7**	100 µl	150 µl	250 µl	

12. Promývání magnetických kuliček

1) Magnetické kuličky se streptavidinem nechat 30 min vytemperovat, promíchat - vortex - 15sec, odpipetovat 50 µl do low binding eppendorf zkumavky 1,5 ml.
2) Dát na magnetický stojánek.
3) Po vyčeření odpipetovat supernatant a vyhodit (nenechat uschnout!max 5min).
4) Přidat 100 µl Bead wash pufr (7), zvortexovat, dát na magnetický stojánek, do vyčeření, supernatant vyhodit.
5) Přidat 100 µl Bead wash pufr (7), zvortexovat, dát na magnetický stojánek, do vyčeření, supernatant vyhodit.
6) Přidat 50 µl Bead wash pufr (7) přendat do 0,2 eppinky, zvortexovat - 10sec, dát na magnetický stojánek do vyčeření, supernatant vyhodit. Pozor – kuličky nesmí vyschnout
7) Přidat celý objem knihovny z cycleru ke kuličkami na magnetickém stojánku a voretxovat - 10sec.
8) Dát do cycleru na 47°C na 15 min. (víko 57°C)
9) Přidat 100 µl 1x wash buffer I (1) k 15 µl Capture bead s navázanou DNA.
10) Vortex 10 sec.
11) Přepipetovat celý objem PCR zkumavky do 1,5 ml eppendrofky low binding.
12) Dát na magnetický stojánek, nechat vyčeřit a odstranit supernatant.
13) Přidat 200 µl 1x Stringent wash pufru (4), voretx 10sec.

14) Inkubovat na 47 °C/ 5 min v termobloku.
15) Dát na magnetický stojánek, nechat vyčeřit a odstranit supernatant.
16) Sundat zkumavku z magnet. stojánku, přidat 200 µl 1x Stringent wash pufru (4) 47°C, 10x pipetováním promíchat nebo 10 sec vortex. Pracovat rychle, aby teplota neklesla pod 47°C.
17) Inkubovat při 47°C/ 5 min v lázni.
18) Dát na magnetický stojánek, nechat vyčistit a odstranit supernatant. Sundat ze stojánku.
19) Přidat 200 µl 1x wash buffer I (1) RT k vzorku a vortexovat 10 sec. Inkubovat 1 min .
20) Na magnetický stojánek, nechat vyčeřit a odstranit supernatant. Sundat ze stojánku.
21) Přidat 200 µl 1x wash buffer II (2) RT k vzorku a vortexovat 10 sec. Inkubovat 1 min.
22) Dát na magnetický stojánek, nechat vyčeřit a odstranit supernatant. Sundat ze stojánku.
23) Přidat 200 µl 1x wash buffer III (3) RT k vzorku a vortexovat 10 sec. Inkubovat 1min.
24) Dát na magnetický stojánek, nechat vyčeřit a odstranit supernatant. Sundat ze stojánku.
25) Přidat 15 µl PCR H_2O o pokojové teplotě k vzorku.

13. Post-Captured LM3-PCR

Z Accessory kitu v2 vyndat:

- Post LM-PCR oligo 1 &2 (LP2, žluté víčko) Při prvním použití přidat 480 µl.
- Kappa hifi hot start ready mix (zelené víčko) – uložit na led nebo do chladítka.

Do 2 PCR zkumavek připravit MIX: 25 µl – Kappa hifi hot start ready mix
5 µl – Post-LM-PCR olig 1,2
20 µl = celá knihovna (pokud je objem menší přidat H_2O)

Vzorek s mastermixem je 10x propipetovat, zásadně. Dát vzorky do cycleru a spustit program LM3 PCR:

Tab. 9: Program LM3 PCR

98°C	45 s	1x
98°C	15 s	14x
60°C	30 s	
72°C	30 s	
72°C	60 s	1x
4°C	∞	

Produkt možno skladovat při teplotě 2 - 8°C až 72 hod.

14. Přečistění LM-PCR magnetickými kuličkami

1) Připravit čerstvý 80% etanol: 400µl ethanolu + 100 µl PCR vody.
2) Do low biding dát 90 µl AMPure Beads a přidat celý PCR produkt (50 µl), vortex 10 sec.
3) Inkubace 5 min.
4) Dát zkumavku na magnetický stojánek, dokud se roztok nevyčeří.
5) Opatrně odsát a vyhodit supernatant.
6) Přidat 200 µl 80% etanolu – spláchnout peletu (nemíchat), nechat 30 s, odsát supernatant, vyhodit.
7) Znovu přidat 200 µl 80% ethanolu – spláchnout peletu (nemíchat), nechat 30 s, odsát supernatant, vyhodit, stočit kuličkami ven, odsát zbyteček ethanolu.
8) Vysušit peletu
9) Resuspendovat peletu v 53 µl H_2O.
10) Inkubace 2 min. při laboratorní teplotě.
11) Dát zkumavku na magnetický stojánek, dokud se roztok nevyčeří.
12) Supernatant opatrně přepipetovat do nové eppendorfky = vzorek 50 µl! Nesmí obsahovat kuličky! Pokud ano, znovu dát na magnetický stojánek a přepipetovat supernatant.
13) Popsat zkumavku „knihovna a datum“, změřit koncentraci na Qubitu a zamrazit při -20°C.

15. Ředění a denaturace knihovny

Ředění knihovny na 2nM

Změřit koncentraci na Qubitu a vypočítat ředění podle tabulky: PHEO výpočet finál. koncentrace-knihovny na 2 nM

Novou 1,5 ml mikrozkumavku označit 2nM, aktuální datum.

K vypočtenému množství přečištěné knihovny přidat odpovídající množství PCR vody.

Ředění knihovny na koncentraci 10 pM a denaturace

1. Připravit čerstvý 0,2M NaOH – do 1,5 ml mikrozkumavky napipetovat 980 µl PCR vody, přidat 20 µl 10M NaOH a důkladně zvortexovat.
2. Naředit 2 nM PhiX Control - do 0,5 ml mikrozkumavky napipetovat 4 µl PCR vody, přidat 1 µl PhiX Control (10 nM) a několikrát protáhnout pipetou.
3. Napipetovat 47,5 µl 2 nM knihovny do 0,5 ml mikrozkumavky a přidat 2,5 µl naředěné 2 nM PhiX Control z kroku 2 a opatrně zvortexovat.

4. Do nové 1,5 ml mikrozkumavky, označené datumem a 10pM, napipetovat 5 µl knihovny s kontrolou z bodu 3., přidat 5 µl 0,2 M NaOH, zvortexovat a krátce stočit.
5. Inkubovat 5 min při laboratorní teplotě.
6. Přidat 990 µl HT1 pufru a dobře promíchat protažením pipetou. Nepoužitý zbytek 10pM knihovny lze uchovávat tři týdny při -20°C.

Takto připravené vzorky jsou analyzovány na přístroji MySeq od firmy Illumina . Získaná data jsou hodnocena pomocí programu FinalistDX od firmy Institute of Applied Biotechnologies.

8.3 Stručný přehled souborů a metod použitých v jednotlivých publikacích

Článek 1:

V tomto článku jsme pro detekci změn v genech *SDHB, SDHD* a *RET* u 13leté probandky použili metodu DGGE a následné sekvenování dle Sangera. Pro analýzu genu *VHL* bylo použito Sangerovo sekvenování. Stejný postup jsme zvolili i pro rodinné příslušníky. Strukturní změny nádorové DNA byly analyzovány pomocí CGH. Pro lokalizaci nádorových ložisek u probandky byla použita metoda CT, MRI a scintigrafie s ^{123}MIBG (Musil et al. 2010).

Článek 2:

Tento článek se zabývá genetickou analýzou PHEO/PGL u dětských pacientů Kliniky dětské hematologie a onkologie 2. LF UK a FN Motol (KDHO). Pro stanovení strukturálních změn chromozomů jsme použili metodu komparativní genové hybridizace (CGH) a array CGH (aCGH), k detekci mutací v genech *SDHB*, *SDHD*, *RET* a VHL jsme použili Sangerovo sekvenování, metoda multiple ligation probe amplification (MLPA) byla použita pro zjištění přítomnosti delecí a duplikací v genech *SDHB, SDHD* a *SDHC* (Vicha A. et al. 2011) .

Článek 3:

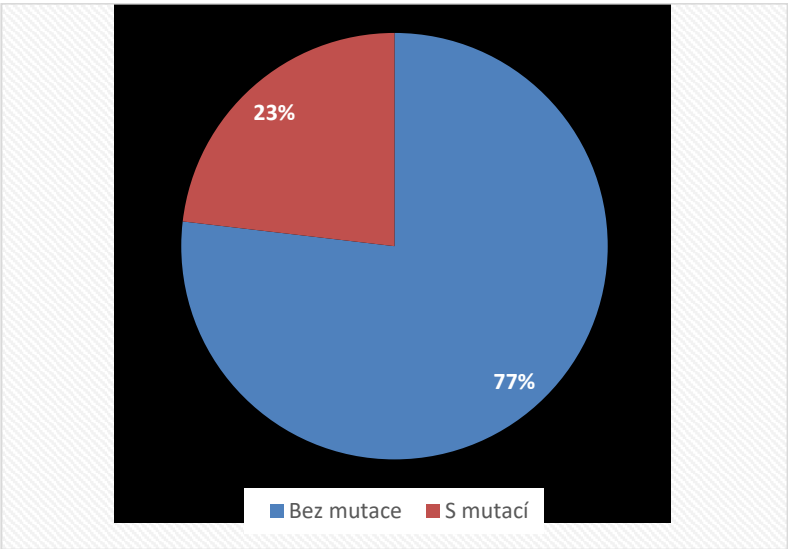
V této práci jsme analyzovali soubor 149 pacientů (41 maligní PHEO, 108 benigní PHEO) z National Institutes of Health (NIH) a z III. Interní kliniky 1. LF UK a VFN. Genetické testování genů *RET, VHL, SDHB* a *SDHD* bylo podle metodiky uvedené v článku (Zelinka T. et al. 2011).

Článek 6:

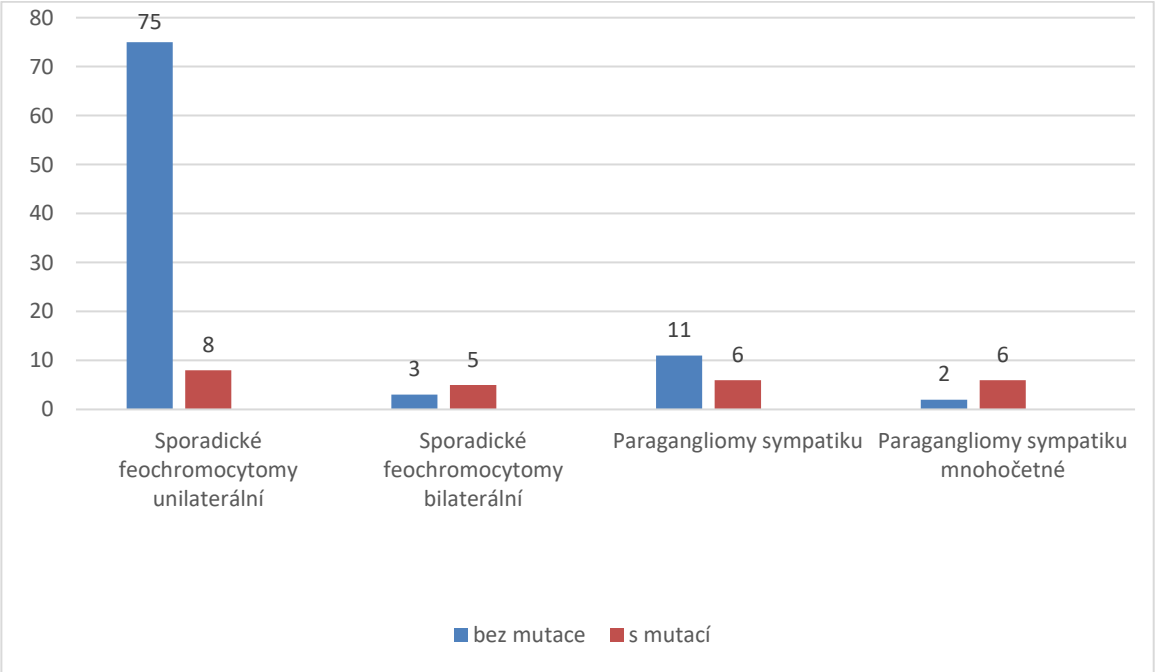
Pro analýzu mutací genu *BRAF* byl použit soubor DNA z nádorové tkáně 64 pacientů s PHEO/PGL (32 mužů a 32 žen, ve věku od 7 do 77 let) z III. Interní kliniky 1. LF UK a VFN a KDHO Motol. Pro detekci mutací jsme použili primery navržené programem Primer3, který je volně dostupný. Pro analýzu real time PCR jsme použili sondy a podmínky dle publikace (Vosecká T. et al. 2017)

9. Výsledky:

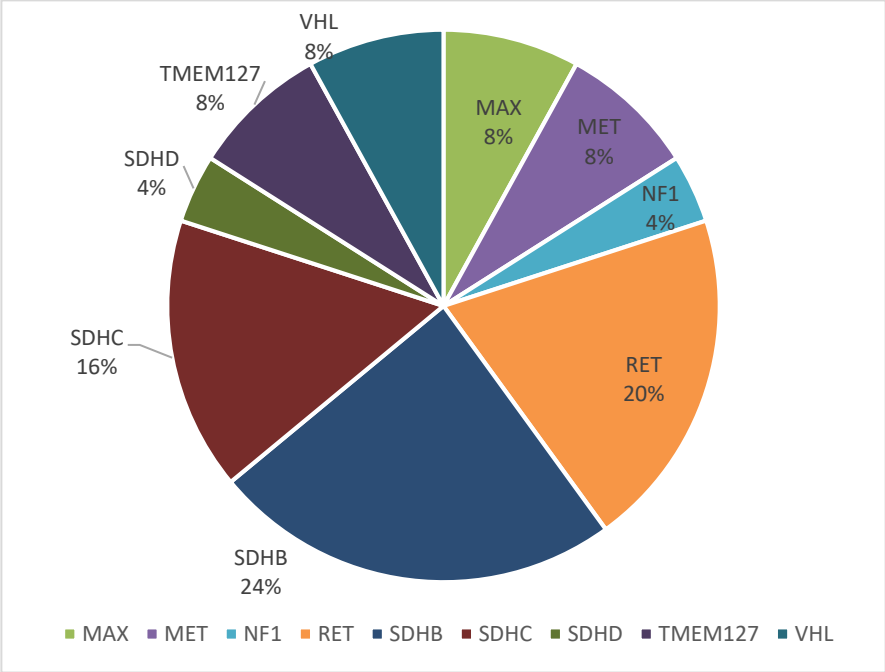
9.1 Výsledky ze souboru analyzovaného metodou NGS



Obr. 3: Zastoupení mutací v celkovém souboru pacientů s PHEO/PGL (doposud nepublikovaná data)



Obr. 4: Zastoupení mutací u jednotlivých klinických kategorií PHEO/PGL (doposud nepublikovaná data)



Obr. 5: Zastoupení mutací u jednotlivých sledovaných genů v souboru pacientů s PHEO/PGL (doposud nepublikovaná data)

9.2 Výstupy disertační práce

9.2.1 Publikace 1

Musil Z., Puchmajerová A., Krepelová A., Vícha A.., Panczak A., Veselá J., Widimský J., Turková H., Lisý J., Kohoutová M.

Paraganglioma in a 13-year-old girl: a novel SDHB gene mutation in the family?

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dle WOS 3x citováno, dle SCOPUS 6x citováno, dle Research Gate 6x citováno

Shrnutí obsahu:

V této publikaci jsme popsali záchyt dosud nepopsané varianty v genu *SDHB*, který kóduje podjednotku enzymu sukcinát-dehydrogenázy. Probandkou byla 13ti letá dívka s příznaky PHEO/PGL tj. hypertenzi, nadměrné pocení a bolestmi hlavy a zvýšenými hodnotami katecholaminů konkrétně norepinefrinu. CT vyšetření objevilo nádorová ložiska v levé paraaortální oblasti (nebo nadledvině) a horním mediastinu. Po odstranění prvního tumoru byla provedena vyšetření pomocí CT, MRI a scintigrafie s 127 iodo-benzylguadininem (^{123}MIBG), která potvrdila nález v horním mediastinu. Následná genetická analýza genů *VHL, SDHB, SDHD a RET* pomocí denaturační gradientové gelové elektroforézy (DGGE) a Sangerova sekvenování odhalila změnu c. 589_600 dup (p. Cys 196 Cys 200 dup.) v 6. exonu genu *SDHB* (ostatní testované geny byly beze změny). Metodika je v článku podrobně popsána. Následná analýza nádorové DNA pomocí CGH odhalila ztrátu v oblasti 1p31.3 , včetně *SDHB* lokusu, ztrátu 11p15.1a zmnožení v oblasti 3p11.1~p13.

Uvedená mutace c. 589_600 dup (p. Cys 196 Cys 200 dup.) je s velkou pravděpodobností zodpovědná za uvedené onemocnění. Domníváme se, že tato varianta ovlivňuje signální dráhu vedoucí ke zvýšené HIF 1A signalizaci. Stejná varianta byla nalezena i u ostaních členů rodiny (viz. rodokmen v článku), kteří byli v době vyšetření bez klinických příznaků. Z tohoto důvodu jsme považovali uvedenou změnu za variantu s nízkou penetrancí, jejíž exprese je ovlivněna hladinami hormonů, růstem v kombinaci s chronickou arteriální hypoxemií způsobenou vrozenou srdeční vadou.

ELSEVIER

Cancer Genetics and Cytogenetics 197 (2010) 189–192

Cancer Genetics and Cytogenetics

Letter to the editor

Paraganglioma in a 13-year-old girl: a novel *SDHB* gene mutation in the family?

Paraganglioma (PGL) is a rare tumor that may be found in the abdomen, the thorax, or the head and neck region. Succinate dehydrogenase (SDH, or succinate-coenzyme Q reductase) was recognized as one of the key molecules playing a role in the pathogenesis of the tumor [1]. An enzyme complex bound to the inner mitochondrial membrane, SDH is the only enzyme that participates in both the citric acid cycle and the mitochondrial electron transport chain. It is a heterotetramer divided into three domains: SDHA, the catalytic domain, SDHB, the electron transfer subunit; and SDHC and SDHD, the anchor subunits.

Germline mutations in the genes encoding the B, D, and C subunits have recently been recognized as associated with paraganglioma syndromes. These three genes are mapped to 1p36.1∼p35 (*SDHB*), 11q23 (*SDHD*), and 1q21 (*SDHC*). Thus, familial PGL syndromes can be classified according to the mutations in individual SDH subunit genes. PGL type 1 is associated with germline mutations in the *SDHD* gene; the tumors are more likely multifocal and rarely malignant than in the other types. The locus for PGL type 2, reported in a large Dutch family, has recently been identified as *SDH5*, on 11q13.1 [2]. The very rare PGL type 3 is associated with mutations in *SDHC*, encoding subunit C of the SDH complex. PGL type 4 is associated with mutations in the *SDHB* gene, and these patients are at high risk for malignant disease [3–5]. Individuals with germline mutations in *SDHB* and *SDHD* may develop tumors along the whole paraganglia distribution area, and they may also be susceptible to pheochromocytoma (PHE) (i.e., intra-adrenal paraganglioma) [6].

The proband, a 13-year-old girl of Czech origin, demonstrated clinical indicators of PGL. She had refractory hypertension, sweating and headaches, and elevated catecholamine concentrations in plasma and urine (namely, norepinephrine). Computed tomography revealed an expansive mass, 35 × 30 × 35 mm) of uncertain etiology in the left para-aortic region (or inside the left suprarenal gland), and an extending mass, 38 × 45 × 50 mm, in the left superior posterior mediastinum region. At surgery, the para-aortic mass was excised and paraganglioma revealed by histology. One month after the first operation, a biopsy of the mediastinal tumor was made and the diagnosis of paraganglioma confirmed. Postoperative computed tomography, positron emission tomography, and magnetic resonance imaging demonstrated voluminous tumor in the superior mediastinum (Fig. 1). Scintigraphy with iodine-123 meta-iodobenzylguanidine (^{123}I-MIBG) also revealed an increased uptake in the same region without any additional foci. The entire tumor was then surgically excised. As of writing, the patient had remained in complete remission for 3.5 years.

The proband was referred to our outpatient clinical genetics department. She had been diagnosed with a defect of the atrial septum at the age of 9 years; her mother had undergone surgery for the same type of congenital heart defect. The proband's father has been treated for glaucoma. There was no family history of neoplastic diseases. Peripheral blood samples were obtained from the proband, all members of her nuclear family, and from her paternal uncle and both of his children.

DNA was extracted using a salting-out method. Mutation analysis of the *VHL*, *SDHB*, *SDHD*, and *RET* genes was performed using polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis. Primers were designed based on GenBank sequences using Primer 3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi); the forward and reverse primers for the exon 6 of *SDHB* were 5′-CCT CTC TTT TCT CCC CAT AC-3′, and 5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC GCA GCA ATC TAT TGT CCT CTT G-3′. The PCR reaction mixture (50 μL) contained 1 × PCR buffer (MBI Fermentas, Vilnius, Lithuania), 50 ng of genomic DNA as template, 1.5 mmol/L $MgCl_2$ (MBI Fermentas), 25 pmol of each primer, 200 μmol/L of each deoxynucleotide triphosphate (Promega, Madison, WI), and 1.0 unit of *Taq*DNA polymerase (MBI Fermentas). Amplification conditions included an initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of 45 seconds at 94 °C, 45 seconds at 58 °C, and 1 minute at 72 °C, with the final extension step running 5 minutes at 72 °C. DNA fragments exhibiting aberrant band shifts were reamplified and sequenced in both directions using an automatic fluorescent ABI Prism 310 genetic analyzer (PE Applied Biosystems) according to the manufacturer's instructions.

A novel nucleotide change, c. 589_600dup (p. Cys196 Cys200dup) in exon 6 of the *SDHB* gene, was found in the proband. The same four-codon duplication was found in her older sister, her father, her paternal uncle, and the

0165-4608/10/$ – see front matter
doi:10.1016/j.cancergencyto.2009.11.010

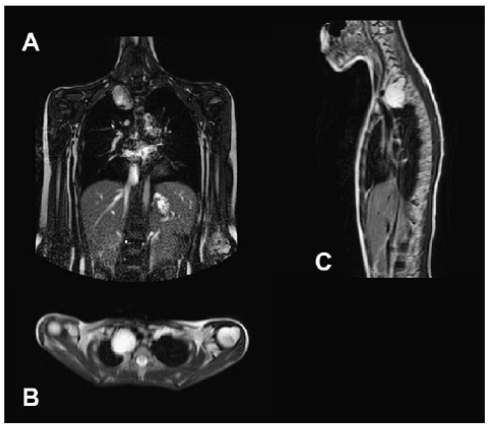

Fig. 1. Magnetic resonance imaging of a voluminous paraganglioma in the upper mediastinum and upper aperture in a 13-year-old girl. (A) sB-TFE sequence in a coronal plane. (B) Transverse section (T_1-SER spin echo). (C) T_2, spin echo sequence in a sagittal plane. The paraganglioma ($38 \times 45 \times 50$ mm) of the right upper mediastinum and upper aperture at the level of C7/Th1−Th4/5 propagates to the right foramen intervertebrale Th1/2 and dislocates adjacent large vessels. The expansion presses the trachea medially and propagates into the dilated right foramen intervertebrale Th1/2, touching the dural sac without compressing it.

uncle's children (Fig. 2). This variant was not found in any of 200 DNA samples from control individuals of both sexes. No *VHL* gene alterations were found in the proband by either sequencing or multiplex ligation-dependent probe amplification analyses, and no mutation was detected in the *SDHD* and *RET* genes.

Comparative genomic hybridization (CGH) was performed to identify chromosome alterations, which may be involved in the development of this tumor. Test DNA was isolated from freshly frozen tumor samples of the patient and reference DNA from healthy volunteers. DNAs were labeled with different fluorochromes by using a commercially available kit (Abbott Molecular, Des Plaines, IL) [7]. Fluorescence imaging and analysis were performed with an Olympus BX51 microscope (Olympus, Tokyo, Japan) and ISIS software (MetaSystems, Altlussheim, Germany), respectively. A chromosomal region was considered to be amplified if the average green-to-red fluorescence ratio exceeded the 1.5 cutoff line (a gain), and the reverse ratio (<0.66) was interpreted as a loss [7]. In addition to the loss of 1p31.3∼ter, including the *SDHB* locus, loss of 11p15.1∼ter and gain of 3p11.1∼p13 were observed in DNA isolated from the proband's tumor.

The mutation detected in exon 6 of the *SDHB* gene is, with high probability, responsible for the multifocal paraganglioma in the proband. Because the same mutation was found in the proband's sister, father, uncle, and two cousins, all of whom show no symptoms of the disease, we can speculate that the penetrance of the mutation is primarily low and its expression influenced by other factors, such as hormonal levels and growth acceleration in the prepubertal 13-year-old girl combined with the chronic arterial hypoxemia due to her congenital heart

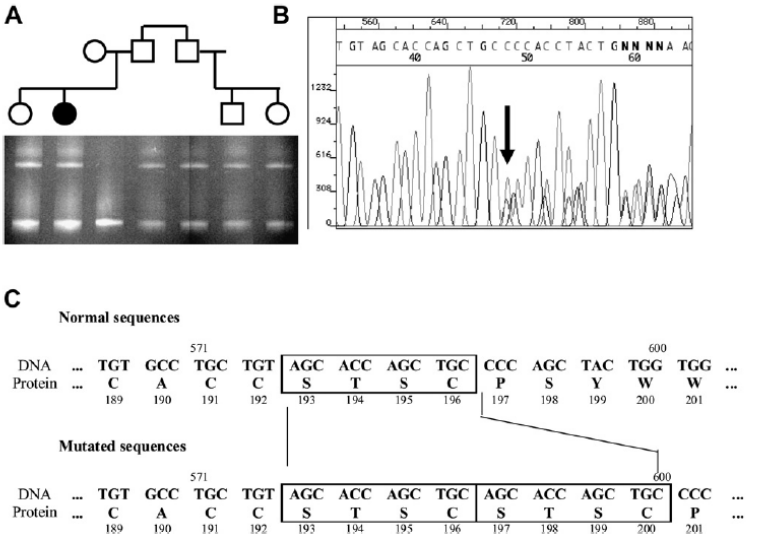

Fig. 2. Molecular diagnostics of the mutation in exon 6 of the *SDHB* gene. (A) Pedigree chart and denaturing gradient gel electrophoresis distribution pattern. Each lane matches the family member directly above it in the pedigree chart. (B) Sequencing of exon 6 of the *SDHB* gene in the proband. (C) Comparison of normal and mutated (duplicated) nucleotide and amino acid sequences.

defect [8]. The mutation is not listed among known alterations of the *SDHB* gene (http://chromium.liacs.nl/lovd_sdh/home). The penetrance for *SDHB* mutations has been recently estimated as 29% by age 30 years and 45% at the age of 40 [6], with a lifetime PGL or PHE expectancy of 76% [8].

The effect of this novel mutation can be judged according to the recent data [1,5,6,9,10]. Germline mutations in *SDHB* coupled with the loss of the wild-type allele in somatic (tumor) cells (as documented in the present case by CGH) are predicted to reduce ubiquinone reductase activity, resulting in accumulation of succinate, which in turn may inhibit prolyl-hydroxylase enzymes and thus lead to increased hypoxia-inducible factor-1 (HIF-1) signaling [1,10]. In the SDH molecule, SDHB is the iron-sulfur subunit required to act together with SDHA protein during catalysis. The SDHB cysteine in position 197 serves as the ligand to the iron-sulfur moiety. Together with proline-198 and surrounding residues (exons 6 and 7), they form the key interface with anchor proteins SDHC and SDHD. Thus, the PHE–PGL susceptibility-associated germline *SDHB* mutations in exon 6 and 7 or truncating mutations before residue 197 are supposed to prevent the assembly of the catalytic complex. The physiological consequence would be a mixture of wild-type enzyme complex and complexes that contain only *SDHC* and *SDHD*, resulting in loss of enzymatic activity [5,9].

In our laboratory, we have 4 years of experience with *SDHB* diagnostics. Among 64 patients with sporadic pheochromocytoma–paraganglioma tested so far, we have found one other *SDHB* mutation, along with many polymorphisms. That mutation (c.423 + 1 G > A), which is recorded in the *SDHB* alterations list of the Leiden Open Variation Database (URL cited above), has been subsequently proved to cosegregate precisely with the disease in an entire family (Z. Musil et al., unpublished data, manuscript in preparation). For the novel four-codon duplication in exon 6 of the *SDHB* gene described here, the estimated penetrance is 1/6, or ~17%. Recently, similarly low penetrance of PGL (11 with PGL, out of 41 mutation carriers) related to the large *SDHB* exon 1 deletion was reported in a family across five generations [11].

The differing phenotypes (i.e., disease vs. healthy status) in individuals sharing the novel mutation may be explained if this mutation is more prone than other *SDHB* mutations to the influence of products of other genes interacting with the SDH complex. There may also be interaction with one or more other tumor-associated genes located on chromosome arms 11 p and 3 p (for which loss and gain, respectively, were documented by CGH).

Only the older sister of the proband has been screened radiologically; the other members of the family sharing the mutation show no symptoms of PGL and to date have not been screened this way. Nevertheless, regular follow-up tests including blood pressure, catecholamines in urine, ultrasonography, computed tomography, and bone scintigraphy are recommended, not only for the proband and her healthy sister, but also for all those who tested positive for the novel mutation, in accordance with the general guidelines from the First International Pheochromocytoma Symposium in 2006 [1,4,6].

Acknowledgments

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9.2.2 Publikace 2

Vicha A., Holzerova M., Krepelova A., **Musil Z.,** Prochazka P., Sumerauer D., Kodet R., Eckschlager T., Jarosova M.

Molecular cytogenetic characterization in four pediatric pheochromocytomas and paragangliomas.

Pathology and oncology research 2011 Dec;17(4):801-8

Impact factor: 1,935

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dle WOS 3x citováno, dle SCOPUS 3x citováno, dle Research Gate 7x citováno

Shrnutí obsahu:

Tento článek je zaměřen na genetickou analýzu dětských pacientů s PHEOchromocytomem a paragangliomem. Pro stanovení chromozomálních změn jsme použili metody komparativní genové hybridizace (CGH) a array CGH (aCGH), k detekci mutací v genech SDHB, SDHD, RET a VHL jsme použili Sangerovo sekvenování, metoda multiple ligation probe amplification (MLPA) byla použita pro zjištění přítomnosti delecí a duplikací v genech *SDHB, SDHD* a *SDHC*. Jednotlivé postupy u použitých metod, klinická data pacientů a výsledky CGH/aCGH jsou popsány v článku.

Chromozomální změny byly nalezeny u všech zkoumaných pacientů. Pacienti s PHEO měli ztráty chromozomu 3 nebo krátkého raménka 3. chromozomu, zatímco u pacientů s paragangliomy jsme objevili ztrátu krátkého raménka 1.chromozomu. Delece na 11. chromozomu byla nalezena u všech pacientů.

V době psaní publikace se byli všichni pacienti v remisi.

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RESEARCH

Molecular Cytogenetic Characterization in Four Pediatric Pheochromocytomas and Paragangliomas

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Abstract Pheochromocytomas (PCCs) are rare tumors among children and adolescents and therefore are not genetically well characterized. The most frequently observed chromosomal changes in PCC are losses of 1p, 3q and/or 3p, 6q, 17p, 11q, 22q, and gains of 9q and 17q. Aberrations involving chromosome 11 are more common in malignant tumors. Unfortunately information about gene aberrations in childhood PCC's is limited. We used comparative genomic hybridization (CGH) and array comparative genomic hybridization (aCGH) to screen for copy number changes in four children suffering from pheochromocytoma or paraganglioma. Patients were diagnosed at the age 13 or 14 years. Bilateral pheochromocytoma was associated with von Hippel-Lindau syndrome (VHL). Multiple paraganglioma was associated with a germline mutation in SDHB. We found very good concordance between the results of CGH and aCGH techniques. Losses were observed more frequently than gains. All cases had a loss of chromosome 11 or 11p. Other aberrations were loss of chromosome 3 and 11 in sporadic pheochromocytoma, and loss of 3p and 11p in pheochromocytoma, which carried the VHL mutation. The deletion of chromosome 1p and other changes were observed in paragangliomas. We conclude that both array CGH and CGH analysis identified similar chromosomal regions involved in tumorigenesis of pheochromocytoma and paragangliomas, but we found 3 discrepancies between the methods. We didn't find any, of the proposed, molecular markers of malignancy in our benign cases and therefore we speculate that molecular cytogenetic examination may be helpful in separating benign and malignant forms in the future.

Keywords Pheochromocytoma · Paraganglioma · Comparative genomic hybridization · Pediatric · Microarray

Abbreviations

CGH	Comparative genomic hybridization
aCGH	Array comparative genomic hybridization
PCCS	Pheochromocytomas
VHL	Von Hippel-Lindau syndrome

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PGL	Paragangliomas
NF1	Neurofibromatosis type 1 gene
SDHB, SDHD and SDHC	The genes encoding the succinate dehydrogenase of mitochondrial subunits B, D, and C
VMA	Vanillylmandelic acid

Introduction

Pheochromocytomas (PCCs) are rare tumors among children and adolescents, despite being the most common pediatric endocrine tumor [1]. They're derived from chromaffin cells that originate from the neural crest. These cells are located in the adrenal medulla (pheochromocytes) and in the paraganglia along the sympathetic chain, and near the aorta. Tumors from extra-adrenal chromaffin tissue are referred to as extra-adrenal pheochromocytomas or paragangliomas (PGL). These two types often share the same clinical course and are histologically equivalent [2, 3]. Twenty percent of all pheochromocytomas occur in childhood [4]. In studies describing pediatric patients, multifocal disease, extra-adrenal disease, and familial association are more frequently described compared to adults. The majority (95%) of pediatric pheochromocytomas are intra-abdominal [5], 40% are bilateral, and 70% are multifocal [4–6]. Although the prevalence of malignancy is commonly cited to be about 10%, other estimates suggest rates of between 3% and 36% of pheochromocytomas/paragangliomas are malignant [7, 8]. Among pediatric patients, approximately 40% of pheochromocytomas are associated with known genetic mutations [9]. At present, the *RET*, *von Hippel-Lindau gene* (*VHL*), *neurofibromatosis type 1 gene* (*NF1*), *succinate dehydrogenase complex assembly factor 1*(*SDHAF1*), and the genes encoding the B, D, and C subunits of mitochondrial succinate dehydrogenase (*SDHB, SDHD* and *SDHC*) are known to be responsible for tumor formation. The chromosomal locations of these genes are summarized in Table 1. Germline mutations in these genes increase the risk of developing pheochromocytomas and/or paragangliomas, which variably associate with other tumors and characterize different clinical syndromes such as Multiple Endocrine Neoplasia 2 (usually MEN 2A, rarely MEN 2B), von Hippel-Lindau (VHL), and NF 1, or the PGL syndromes. The *SDHB* mutation predisposes patients to extra-adrenal locations and metastatic disease and has been more frequently reported in children [1, 10–12].

Due to the frequency of PCCs in childhood, it has been hypothesized that germline mutations in *RET*, *VHL*, *SDHB*, and *SDHD* cause PCCs more frequently among pediatric patients [9, 13, 14].

Table 1 Characteristics of genes associated with familial forms of pheochromocytoma

Gene	Chromosome	Protein
VHL	3p25-26	pVHL19 and pVHL30
SDHB	1p36.13	Catalytic iron-sulfur protein
SDHD	11q23	CybS(membrane-spanning subunit)
SDHC	1q21	CybL (Large subunit)
SDHAF1	19q13.12	Assembly factor 1
RET	10q11.2	Tyrosine-kinase receptor
NF1	17q11.2	Neurofibromin

A major problem in PCC relates to the unpredictability of clinical outcomes. Presently there are no defined histological markers to differentiate between benign and malignant PCCs. Features which arouse suspicion of malignancy include large tumor size, small tumor cells, extensive necrosis, vascular invasion, and aneuploidy [15–17]. Only the presence of distant metastases, derived from large pleomorphic chromaffin cells, is widely accepted as a criterion of malignancy [18]. The most frequently observed chromosomal changes in PCC are losses of 1p, 3q and/or 3p, 6q, 17p, 11q, 22q, and gains of 9q and 17q. Aberrations involving chromosome 11 are more common in malignant tumors. Unfortunately, information about gene aberrations in childhood PCC's is limited [19–22].

Applying chromosome comparative genomic hybridization (CGH), we first screened tumor specimens from four pediatric patients to identify genomic aberrations. Next, we validated these findings using array comparative genomic hybridization (aCGH) to increase mapping resolution. This was done because CGH resolution is limited to 10–20 Mb. When we compared results from both techniques, we found some discrepancies. These discrepancies can't be completely explained by limited resolution, indicating that tumor heterogeneity played a role in the discrepancies observed in our results.

Patients and Methods

A total of four cases were diagnosed and treated in the Department of Pediatric Hematology and Oncology of Motol University Hospital, 2nd Medical Faculty of Charles University, Prague, CZ, between 2003 and 2005.

Case Histories

Case 1

An asymptomatic 13-year-old boy was referred to our hospital for hypertension (blood pressure 190/110 mmHg). The

physical examination was entirely normal except for hypertension. A computed tomography (CT) scan examination revealed bilateral adrenal masses (left, 4 cm×3.5 cm×5 cm; right, 5 cm×4 cm×4.5 cm). Biochemical investigation showed an elevated 24-hour urine vanillylmandelic acid (VMA) level of 52.2 mmol/mol of creatinine per day (normal range 0.4–4.0 mmol/mol of creatinine per day). Fundoscopic examination revealed a capillary angioma.

A bilateral resection of the adrenal glands was performed and histological examination of the tissues confirmed pheochromocytoma. Von Hipple-Lindau syndrome was confirmed, DNA sequence analysis revealed a novel germline, heterozygous transversion M_000551:c.374A>C (p.His125Pro) in exon 2 of the *VHL* gene. The mother of the patient was negative for the *VHL* mutation; the father was not examined. The family history was negative for VHL syndrome and PCCs. The patient remains in complete remission (CR) 79 months after diagnosis.

Case 2

A 14-year-old boy presented to the emergency department with a history of a single, 2 min, episode of syncope with trismus. He was found, incidentally, to be hypertensive (blood pressure, 160/100 mmHg) with a history of headaches, fatigue, and vomiting. An abdominal CT scan revealed a mass on the right adrenal gland (4 cm× 4.2 cm×4 cm). Biochemical investigation showed an elevated 24-hour urine VMA level of 19.2 mmol/mol of creatinine per day (normal range 0.4–4.0 mmol/mol of creatinine per day). A right adrenal gland resection was performed. Histopathological examination of the tissue confirmed pheochromocytoma.

A germline mutation of the *VHL* gene wasn't identified in this case; however, we found a new somatic heterozygous mutation in the second exon of the *VHL* gene c.389 T>G (p. Val130Gly). We use the Multiplex Ligation-dependent Probe Amplification (MLPA) method to screen for large gene deletions in the VHL and SDH genes. This method was used because large gene deletions account for a considerable proportion of PCC syndromes. A large deletion in the VHL and SDH gene wasn't identified in this case. The family history was negative for VHL syndrome and PCCs. The patient remains in CR 57 months after diagnosis.

Case 3

A routine, preventive care, examination by a local pediatrician of a 13-year old boy revealed palpated resistance in the abdomen. A CT scan showed a spherical tumor on the left side of the abdomen (10 cm×8 cm×10 cm) with small local calcifications and hemorrhagic necrosis. Blood pressure was 95/50 mmHg. Twenty-four hour urine VMA was within normal range. A total surgical resection of the tumor was performed. Pathological examination of the tumor tissue confirmed paraganglioma. No germline or somatic mutation of *VHL*, *RET*, *SDHB*, or *SDHD* were found. MLPA was used for detecting large gene deletions in the VHL and SDH genes. While a large deletion in the VHL and SDH genes wasn't identified in peripheral blood, we found loss of one copy of SDHB, and gain of SDHC in the tumor tissue. The patient remains in CR 53 months after diagnosis. The family history was negative for PCCs.

Case 4

A 13-year-old girl was examined for a 3 year history of, unilateral (right side), sweating. Over the last 2 years she had suffered from headaches with vomiting two to three times per month. She was found to have hematuria and proteinuria, anisocoria, acute hypertension retinopathy, and hypertension (blood pressure, 223/153 mmHg). The child was referred to pediatric oncology with hypertension and a palpable abdominal mass. CT scan of chest and abdomen revealed a mediastinal mass (4.5 cm×4.5 cm×4.5 cm) and a left retroperitoneal mass (3.5 cm×3 cm×3.5 cm). A total surgical resection of both tumors was performed. The histopathological examination of the two lesions confirmed paraganglioma in both tumors. A germline heterozygous mutation in the *SDHB* gene was identified as *SDHB* 6 c.589 600 dup (p.Cys 196 Cys 200 dup) [23]. The same four-codon duplication was found in her older sister, her father, her paternal uncle, and the uncle's children. All of them were without history of any neoplastic disease. The patient remains in CR 49 months after diagnosis.

Methods

Comparative Genomic Hybridization

Tested DNA was extracted from fresh frozen samples to reference DNA came from 20 male to 20 female peripheral blood samples of healthy volunteers. Isolated DNA was mixed together (male or female). DNA was labeled with different fluorochromes using a commercially available kit and carried out according to the manufacturer's instructions (Abbott Molecular; Abbott Park, Illinois, U.S.A.), with a minor modification [24]. Fluorescence imaging and analysis were performed using an Olympus BX51 microscope (Olympus; Tokyo, Japan) and ISIS software (MetaSystems; Altlussheim, Germany). Thirty metaphases were captured and analyzed from each sample. Chromosomal regions were considered to be over-represented if the average green-to-red fluorescence ratio exceeded a cutoff of 1.25

(again) and as under-represented if the ratio was below a cutoff of 0.75 (a loss).

Array Comparative Genomic Hybridization

We used a commercially available genomic DNA microarray kit (GenoSensor Array 300; Abbott Molecular), which contained DNA representing 287 genes from the BAC, PAC, to P1 libraries. Each cloned DNA was spotted on slides, in triplicate. CGH was performed according to manufacturer's instructions (Abbott-Molecular) and analyzed with a microarray reader and analysis software (GenoSensor Array 300 system, Abbott-Molecular). Spots with G/R ratios more than the mean plus two standard deviations (≈1.2) were considered as gains, while spots with G/R ratios less than the mean minus two standards deviations (0.8) were considered as losses in copy number.

Gene Analyses

Genomic DNA was extracted from peripheral leukocytes or tumor cells using a salting out method modified according to Miller et al. (1988). We amplified exons 10, 11, and 13 through 16 of the *RET* proto-oncogene according to Neumann et al. (2002). Mutation analysis was carried out using DGGE (Denaturing Gradient Gel Electrophoresis) as previously described [25].

The six exons of the *RET* were amplified in a 25 μl reaction volume with 0.5 μM of each primer, 1x PCR buffer, 1.5 mM $MgCl_2$, 100 μM of each dNTP, between 50 and 300 ng of genomic DNA (as a template), and 1 unit of Taq DNA polymerase. PCR was performed for 35 cycles (30 s; 94°C, 45 s; 57–62°C, 40 s; 72°C) with a final extension of 10 min at 72°C. DGGE conditions are available on request. DNA fragments with an aberrant shift on DGGE were analyzed on an automatic fluorescent ABI Prism™ 310 Genetic Analyzer (PE Applied Biosystems) according to the manufacturer's instructions.

Exons 1 to 3 of the *VHL* gene and exon-intron boundaries were amplified (primer sequences available on request). PCR was performed in a 30 μl reaction containing 1x PCR buffer, 1.0 mM $MgCl_2$, 100 μM of each dNTP, 0.5 μM of each primer, and 1 unit of Taq DNA polymerase (Fermentas, Lithuania). The PCR conditions were as follows: 32 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 58°C and extension for 2 min at 72°C, followed by a final extension for 7 min at 72°C. PCR products were then purified using Quick-Clean purification solution (Bioline), and both forward and reverse strands were sequenced using the appropriate PCR-primer and BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed on a ABI Prism 3100 Avant Genetic Analyzer (PE BioSystems).

Eight *SDHB* exons and four *SDHD* exons were screened using DGGE. Primers were designed based on GenBank sequences using Primer 3 software (available at: http://www.hgmp.mrc.ac.uk/GenomeWeb/nuc-primer.html) including intron-exon boundaries. The melting profile of DNA fragments, location of primers and GC clamps were analyzed using MacMelt™ software (Bio-Rad, California). The PCR reaction mixture (50 μl) contained 1x PCR buffer (MBI Fermentas), between 50 and 300 ng of genomic DNA (as template), 1.5 mM $MgCl_2$ (MBI Fermentas), 25 pmol of each primer, 200 μM of each deoxynucleotide triphosphate (Promega, USA), and 1.0 units of *Taq*DNA polymerase (MBI Fermentas). The amplification conditions included an initial denaturation at 94°C for 3 min, followed by 35 cycles of 45 s at 94°C, 45 s at 55–65°C (optimal annealing temperature according to the primers conditions), 1 min at 72°C and final extension step running for 5 min at 72°C. DNA fragments exhibiting aberrant band shifts were re-amplified and sequenced in both directions using an automatic fluorescent ABI Prism™ 310 Genetic Analyzer (PE Applied Biosystems) according to the manufacturer's instructions.

MLPA

Multiplex Ligation-dependent Probe Amplification (MLPA) analysis was used to detect deletions or duplications in the *VHL* or *SDHB, SDHC, SDHD* and *SDHAF1* genes. The SALSA MLPA P016B *VHL* and the P226-B1 SDHD probe kits (MRC-Holland, Amsterdam, Netherlands) were used. The P016B kit contains eight probes to the *VHL* gene (four in exon 1, two in exon 2 and two in exon 3), additional probes to other genes on 3p and control probes to regions telomeric and centromeric from *VHL*. The P226-B1 kit contains nine probes to *SDHB,* seven probes to *SDHC,* five probes to *SDHD* and one probe to *SDHAF1*. Detailed information on probe sequences, gene loci and chromosome locations can be found at www.mlpa.com.

Genomic DNA (50–200 ng) was denatured and the probes were allowed to hybridize (16 h at 60°C). PCR was performed on the samples in a volume of 50 μl containing 10 μl of the ligation reaction mixture and using a thermal cycler Mastercycler ep gradient (Eppendor, Hamburg, Germany). Aliquots of 1 μl of the PCR reaction were combined with 0.5 μl ROX-labelled internal size standard (Applied Biosystems, Foster City, CA, USA) and 12 μl deionized formamide. Fragments were separated by electrophoresis on an Applied 3130XL capillary sequencer and quantified using GeneMarker version 1.6 software (SoftGenetics). For copy-number detection, normal control DNA samples were included in each set of MLPA experiments. Interpretation was based on the comparison

of peak heights between the control DNA and the tumor sample. Cut-off levels for loss of relative copy number were set at 0.75.

Results

Clinical data and CGH/aCGH results are summarized in Table 2 and Fig. 1. Chromosomal imbalances were observed in all 4 cases. The average amount of genetic aberrations was CGH/aCGH 2.75 and 2.5 changes, respectively (range 2–4) per case. Losses were as common as gains. A comparison of our CGH/aCGH data with data from adult to pediatric patients reported in the literature, together with the Progenetix CGH database (http://www.progenetix.net/progenetix/;14.9. 2009) showed high concordance of the aberration pattern [19–21].

A deletion on chromosome 11 was found in all cases (3×11p, 1×11). Patients with pheochromocytoma showed an aberration pattern distinct from paraganglioma patients. In both pheochromocytomas, there were losses of chromosome 3 or 3p. Paragangliomas showed losses of 1p. In case 4, aCGH detected a 1p deletion, while CGH only detected a partial deletion (1p31.3-ter). Patient 2 had a deletion on chromosome 3 and 11; we didn't find a constitutive *VHL* point mutation, using DNA sequencing, in the DNA extracted from peripheral blood leukocytes. Therefore, we sequenced the DNA from the tumor tissue to detected a new *VHL* mutation, *VHL* c.389 T>G (p. Val130Gly). The patient with the largest tumor, 416 cm^3 (case 3), showed the most extensive genetic changes including deletion of 17p11.2-pter and gain of 1q11-qter. In this case, we didn't find a constitutive or somatic mutation. In case 4, we found a discrepancy between CGH and aCGH results on chromosome 17. Therefore, fluorescence in situ hybridization (FISH) was used for validation of these results. We used ON *p53* (17p13)/*MPO* (17q22) ISO 17q″ probe (Kreatech Diagnostic) and *RARA* (17q21.1) probes. Testing of nuclei showed diploid status in 45% of *RARA* and 37% of *p53/MPO*. In the majority of nuclei there was aneuploidy, tetraploidy (*RARA* 39%, *p53/MPO* 20.5%), and triploidy (*RAR* 5%, *p53/MPO* 12%). An imbalance was detected in only 25% of nuclei (*MPO/p53*). This is probably due to the heterogeneity of the tumor cell population [26]. Another discrepancy between the techniques utilized in this study was found on chromosome 13, where CGH detected a gain of 13q13-q24, which was at odds with aCGH results. The difference might be explained by a low density of genes, on the chip, at chromosome 13.

We did not observe any correlation between genomic changes and prognosis of the disease. All patients are in the first complete remission following surgical treatment with a median follow up of 55 months.

Table 2 Clinical and genetic data

Case	Patient data			Tumor characteristic		CGH/aCGH imbalances			MLPA	Follow- up (year)
	Sex/age (year)	Germinal mutation	somatic mutation	size (cm^3)	Origin	Loss		Gain		
1	m/13	Het.-*VHL* c.374A>C	n.d	36÷47	pheochromocytoma	CGH	3p12-pter;11p11-pter	–	n.d.	CR (6.58)
						aCGH	3p14.2-p26; 11p15.5-pter	–		
2	m/14	neg.	Het.-VHL c.389 T>G	35	pheochromocytoma	CGH	3;11	–	GGA. negative	CR (4.75)
						aCGH	3;11	–		
3	m/13	neg.	neg.	416	paraganglioma	CGH	1p11-pter;11p11-pter;17p12-pter	1q11-qter	GGA negative	CR (4.41)
						aCGH	1p13.1-pter;11p13-pter, 17p11.2-pter	1q11-qter	SGA del SDHB; gain SDHC	
4	f/13	Het.- *SDHB* 6 c.589-600dup. AGC ACC AGC TGC	n.d	47÷19	paraganglioma	CGH	1p31.3-pter; 11p15.1-pter;17p11.2-ter[a]	13q13-q24 17p[a]	n.d.	CR (4.00)
						aCGH	1p12-pter; 11p13-pter			

n.d. not done, *neg.* negative, *GGA* germinal gene ateration, *SGA.* somatic gene alteration, *CR* complete remission, *m* male, *f* female [a] FISH found heterogeneity in status of 17p, *Het.* heterozygous

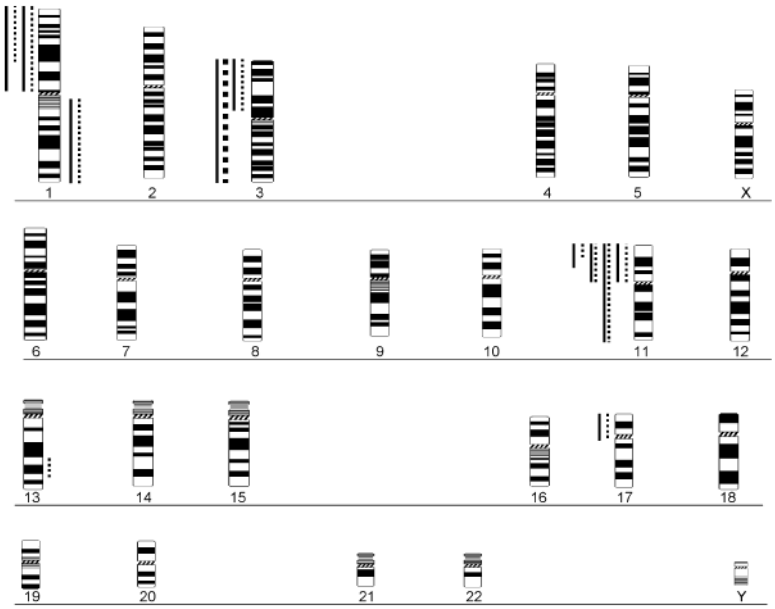

Fig. 1 Frequency plot of genetic changes for all 4 PCC. Loss and gain of chromosomal material are depicted by vertical bars to the left (loss) and right (gain) of chromosomes, respectively. *Dashed lines* indicate chromosomal CGH and normal lines aCGH. A deletion on chromosome 11 was found in all cases (3×11p, 1×11). Patients with pheochromocytoma showed an aberration pattern distinct from paraganglioma patients. In both pheochromocytomas, there were losses of chromosome 3 or 3p. Paragangliomas showed losses of 1p

Discussion

In agreement with other studies [4, 19–22], we found unbalanced chromosomal aberrations in PCCs, using CGH. This suggests that chromosomal changes might be an important tumorigenic event. Data from the literature shows that the most common copy number changes in PCC include loss of chromosomes 1p, 3q, 3p, 11p,11q, 6q, 17p, 22 and gain of chromosome 9q, 17q, and 20q [19–22, 27]. In our pediatric study, the most commonly observed chromosomal imbalances in PCCs included 1p, 3p, and 11p. All cases had more than one unbalanced change. These findings support the hypothesis of Koshla et al. [28] regarding involvement of multiple genes in the pathogenesis of these tumors.

Lui et al. [22], in a study of adult patients with PCC, reported a strong association between *VHL* mutations and loss of chromosomes 3 and 11. Hering's and our data suggest that mutations in *VHL*, which are either hereditary or somatic in origin, are also associated with 3p and 11p deletions. Hering et al. [21] identified a combined deletion of 3p and 11p in only 40% and combined deletion 3 and 11 in the remaining cases of VHL-associated PCC. In our study, we found loss of chromosome 11 or 11p in all cases (*VHL*-related pheochromocytoma and also in paraganglioma). Dannenberg et al. [19] detected the loss of 11p in two out of nine sporadic paragangliomas using CGH. Furthermore, loss of 11p has been reported in 5 of 11 sporadic abdominal paragangliomas [20]. We also detected deletion of 11p in a case of abdominal paraganglioma involving a mutation of the SDHB gene.

Numerous cases of deletion of chromosome 11 or 11p support the hypothesis that genes, relevant to PCC, are on the p arm of chromosome 11. Potential candidate genes are numerous and include WT1, IGF2, BW1, CDKN1C, H19 and others. Imprinting effects are important in some of these genes [21, 29].

Malignant pheochromocytomas represent very rare childhood tumors. Older age, absence of genetic syndromes in the family history, and DNA diploid tumors are favorable, relative to outcomes in pediatric PCC. The distinction between benign and malignant PCC cannot be made on the basis of clinical, biochemical, or histopathologic characteristics [4]. Data on genetic events, which could determine the malignant potential of PCCs are, so far, unsatisfactory, but some chromosomal changes (deletion 11q22-qter, deletion 6q) and aneuploidy are found more often in malignant tumors.

Edström et al. [20] showed that the main difference between benign and malignant tumors was partial deletion or gain of chromosome 11, as observed in 9 out of 12 malignant cases and 3 of 16 benign tumors. Among nine patients which developed metastasis, eight showed involvement of chromosome 11. Loss of 11q22-23 was significantly more common in malignant tumors than in benign ones [20]. Deletion of 11q22-23 has been described, by Hering, in patients with metastatic disease, which might strongly suggest the malignant potential of PCCs [21].

None of the tumors in our study showed loss of 11q22-qter as a solo aberration.

Frequent allelic imbalances at 6q have been reported in other malignancies and appear to be related to a poor prognosis or metastatic disease [30–32]. Dennenberg et al. [19] detected a loss of 6q in 34% of sporadic pheochromocytomas. These deletions were strongly associated with metastatic disease, although, Lemeta et al. [3] found that 72% of pheochromocytomas, including tumors classified as either benign or borderline, showed allele loss at 6q in two commonly affected regions (6q14 and 6q23-24). All cases were sporadic PCCs and the authors didn't find any significant difference in the allele loss between benign and borderline tumors. August et al. [33] was unable to confirm that a loss of 6q was an important event in tumor progression. CGH and aCGH did not revealed chromosomal changes on chromosome 6 in our cases.

Gain of genetic material is more frequently associated with malignant courses. The total number of genetic aberrations is higher in malignant tumors compared to benign tumors. Edström et al. [20] found a wide range in a number of genetic aberrations in both malignant tumors (mean = 6) and benign tumors (mean = 2.5). Dannenberg et al. [19] observed only a marginal association between the mean number of chromosomal alterations and malignancy (5.3±2.7 versus 8.2±6.1). August et al. [33] showed that tumors with 10 or more copy number changes were always associated with the development of metastases at a later stage, the presence of 8 chromosomal aberrations was associated with the occurrence of metastases in 85% of cases, while 60% of metastatic tumors showed less than 6 chromosomal aberrations. In our study the average of genetic aberrations, as revealed by CGH/aCGH, was 2.75 and 2.5 (range 2–4) per case, respectively. Additionally, we didn't find any of the 'supposed' molecular markers of malignancy in our patients.

In conclusion, our results showed which copy number changes, were the most common copy number changes in PCC's. Regarding the most common changes (1p, 3, 3p, 11, 11p), both techniques yielded similar results, however, we found 3 discrepancies between the methods. These discrepancies can't be completely explained by limited resolution, indicating that tumor heterogeneity played a role in the discrepancies observed in our results.

Our observations lead us to suggest that the incidence of deletion of chromosome 11 or 11p is more common in childhood PCC, than in adult PCC. These copy number alterations may play a significant role in PPC tumorigenesis.

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9.2.3 Publikace 3

Zelinka T., **Musil Z.,** Dušková J., Burton D., Merino M.J., Milosevic D., Widimský J. Jr, Pacák K.

Metastatic pheochromocytoma: does the size and age matter?

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Shrnutí obsahu:

Tato publikace shrnuje výsledky studie zaměřené na popis klinických, genetických a histopatologických znaků odlišující maligní a benigní formy PHEOchromocytomu. Na souboru 149 pacientů (41 maligní PHEO, 108 benigní PHEO) jsme hodnotili velikost a biochemický profil primárního nádoru, věk pacientů a čas do rozvoje metastáz.

Pacienti s maligním PHEO byli diagnostikováni v mladším věku (41,4 ± 14,7 vs. 50,2 ± 13,7), rozměr primárního nádoru byl také větší (8,38 ± 3,27 cm vs. 6,18 ± 2,75 cm) a častěji sekretovali norepinefrin (95,1% vs. 83,3%). Signifikantní rozdíly nebyly pozorovány ve výskytu genetických mutací (25,7 % u metastatických PHEO a 14,7 % u benigních PHEO). Při hodnocení histopatologických znaků pouze nekróza se vyskytovala častěji u metastatických PHEO (27,6 % vs. 0 %). K nálezu metastáz došlo v mediánu po 3,6 letech, nejdelší interval byl 24 let.

Závěrem této publikace můžeme říci, že bez ohledu na genetické pozadí, velikost a věk pacienta v době primárního nádoru jsou dva nezávislé faktory pro rozvoj metastatického PHEO.

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ORIGINAL ARTICLE

Metastatic pheochromocytoma: Does the size and age matter?

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ABSTRACT

Background Pheochromocytomas are tumours arising from chromaffin tissue located in the adrenal medulla associated with typical symptoms and signs which may occasionally develop metastases, which are defined as the presence of tumour cells at sites where these cells are not found. This retrospective analysis was focused on clinical, genetic and histopathologic characteristics of primary metastatic versus primary benign pheochromocytomas.

Materials and methods We identified 41 subjects with metastatic pheochromocytoma and 108 subjects with apparently benign pheochromocytoma. We assessed dimension and biochemical profile of the primary tumour, age at presentation and time to develop metastases.

Results Subjects with metastatic pheochromocytoma presented at a significantly younger age (41·4 ± 14·7 vs. 50·2 ± 13·7 years; $P < 0·001$) with larger primary tumours (8·38 ± 3·27 vs. 6·18 ± 2·75 cm; $P < 0·001$) and secreted more frequently norepinephrine (95·1% vs. 83·3%; $P = 0·046$) compared to subjects with apparently benign pheochromocytomas. No significant differences were found in the incidence of genetic mutations in both groups of subjects (25·7% in the metastatic group and 14·7% in the benign group; $P = 0·13$). From available histopathologic markers of potential malignancy, only necrosis occurred more frequently in subjects with metastatic pheochromocytoma (27·6% vs. 0%; $P < 0·001$). The median time to develop metastases was 3·6 years with the longest interval 24 years.

Conclusions In conclusion, regardless of a genetic background, the size of a primary pheochromocytoma and age of its first presentation are two independent risk factors associated with the development of metastatic disease.

Keywords Epinephrine, malignant pheochromocytoma, norepinephrine.

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Introduction

Pheochromocytomas and paragangliomas are tumours arising from chromaffin tissue located either in the adrenal medulla (pheochromocytomas) or at extra-adrenal sites along the sympathetic and/or the parasympathetic chain (paragangliomas) [1,2]. These tumours typically occur in a benign form but may present in a malignant form and reach up to 36% or even higher in subjects with extra-adrenal tumours, in particular when associated with the mutation of the gene encoding the B subunit of the mitochondrial complex II enzyme succinate dehydrogenase enzyme (*SDHB*) [3–8]. Metastatic pheochromocytoma or paraganglioma is defined as the presence of tumour cells at the sites where these cells are not found such as lymph nodes, bones, liver or lungs [9].

To predict the likelihood of metastatic spread, several criteria and scoring systems have been developed on the basis of histopathology or clinical data, but no system has proven reliable [10]. For example, a multiparameter Pheochromocytoma of the Adrenal Gland Scaled Score (PASS), which includes several

histopathologic features of the primary tumour such as mitotic activity and capsular or vascular invasion [11], has been shown unreliable because of its high interobserver and intraobserver variation [12]. From several clinical studies, the highest predictive value might include the extra-adrenal tumour location, presence of *SDHB* mutations and tumour size [4,13,14]. Most of the studies focused on predictors of malignancy in pheochromocytomas or paragangliomas have not addressed adrenal or extra-adrenal tumours separately on a large scale because of their rarity [15,16].

In the study of O'Riordain *et al.*, [5] subjects with paragangliomas that had tumours larger than 5 cm showed a worse prognosis compared to those that had smaller tumours. In the study of Amar *et al.*, [3] the smallest tumour diameter of subjects with malignant pheochromocytoma and paraganglioma was 5 cm, and Kimura *et al.* [17] showed that malignant adrenal pheochromocytoma was significantly larger than tumours of subjects with benign adrenal pheochromocytomas. In contrast, Thompson [11] found no significant difference in the tumour diameter between benign and metastatic pheochromocytoma.

In the present study, we retrospectively assessed clinical, genetic and histopathologic characteristics of primary metastatic from primary benign adrenal pheochromocytomas. In particular, we focused on common histopathologic characteristics including the tumour dimension, the presence of necrosis, capsular, vascular, and lymphatic invasion and genetic background as possible risk factors of their metastatic behaviour. Patients presenting with metastatic paragangliomas (extra-adrenal tumours) were not included.

Subjects and methods

Patients

We identified 41 subjects with metastatic pheochromocytoma referred to either the National Institutes of Health (NIH), Bethesda, USA, or to the 3rd Department of Medicine, General University Hospital, Prague, Czech Republic. Four additional subjects were not included in the analysis because the size of their primary tumours was not available. Metastasis was defined as the presence of tumour cells at sites where no chromaffin tissue is normally found and the metastatic lesion(s) were proven either by biopsy or the presence of persistently elevated catecholamine/metanephrine levels together with a positivity on pheochromocytoma specific imaging modalities and previous history of pheochromocytoma [9]. As the control group, we used 108 subjects with apparently benign pheochromocytoma with the average follow up of 5·8 ± 4·3 years. Study protocols were approved by the Institutional Review Board of the National Institute of Child Health and Human Development at NIH and by the Ethics Committee of the First Faculty of Medicine. All patients provided written informed consent. Reporting of the study conforms to STROBE along with references to STROBE and the broader EQUATOR guidelines [18].

Biochemical phenotype of tumours was characterized according to elevations of particular catecholamines (epinephrine, norepinephrine and dopamine) or their metabolites (metanephrine and normetanephrine) either in urine or in plasma. All elevations above the upper range of normal values were counted as positive and further detailed work-up was initiated. In most subjects, elevations of plasma or urinary catecholamines or metanephrines were many times above upper reference limits. Genetic testing for mutations in pheochromocytoma susceptible genes [rearranged during transfection (*RET*), von Hippel–Lindau gene (*VHL*), *SDHB* and succinate dehydrogenase subunit D gene (*SDHD*)] was performed at NIH, at Mayo Medical Laboratories, Rochester, MN, or at Division of Molecular Diagnostics at the University of Pittsburgh Medical Center Department of Genetics of the Children's Hospital of Philadelphia, PA, as described elsewhere [6] or at Institute of Biology and Medical Genetics of the First Faculty of Medicine, Charles University in Prague. In total, 35 (85%) subjects with metastatic pheochromocytoma and 75 (69%) subjects with benign pheochromocytoma completed genetic testing. Diagnosis of neurofibromatosis type I was made based on a clinical presentation. Certain histopathologic parameters of potential tumour malignancy (vascular, lymphatic and capsular invasion, increased mitotic figures and necrosis) were obtained from available pathological reports [(29 (71%) subjects with metastatic pheochromocytoma and 108 (100%) subjects with benign pheochromocytoma].

Data are shown as mean ± standard deviation (SD) or median and interquartile ranges in case of nonparametric data distribution. Continuous variables were compared using the unpaired *t*-test and categorical variables by the Fisher exact test. Tumour dimension was assessed using the longest tumour diameter. Kaplan–Meier method was used to estimate the time from diagnosis of pheochromocytoma to diagnosis of malignancy and to estimate survival of subjects with metastatic pheochromocytoma, where survival was defined as the time from diagnosis of pheochromocytoma to the date of death or date of last follow-up. We used log-rank test to compare survival between subgroups of patients. $P < 0{\cdot}05$ values were considered as significant. Data were analysed using the statistical software package Statistica version 9.1CZ (StatSoft, Tulsa, OK, USA).

Results

In total, we analysed 41 subjects with metastatic adrenal pheochromocytoma and 108 subjects with benign adrenal pheochromocytoma. Primary tumours of subjects with malignant pheochromocytoma were diagnosed at a significantly

younger age (41·4 ± 14·7 vs. 50·2 ± 13·7 years; $P < 0·001$) compared to subjects with benign pheochromocytomas ($P < 0·001$) (Table 1). No significant difference was found in gender distribution between subjects with malignant and benign adrenal pheochromocytomas, although male subjects tended to present more frequent metastases (Table 1). Among available histopathologic parameters, only necrosis occurred significantly more frequently in the metastatic pheochromocytoma group ($P < 0·001$) (Table 2).

Genetic testing for germ-line mutations or clinical assessment revealed 9 (25·7%) carriers of germ-line mutations in patients with malignant pheochromocytoma (2 × *SDHB*, 4 × *RET* and 3 × *VHL*), compared to 11 (14·7%) carriers in subjects with benign pheochromocytoma (2 × *RET*, 5 × *VHL* and 4 × neurofibromatosis type I) (Table 1). No mutation of the *SDHD* gene was found.

Primary pheochromocytomas in subjects with metastatic spread were significantly larger than in subjects with benign pheochromocytomas ($P < 0·001$). The distribution of tumour dimensions was variable ranging from 2·4 to 17 cm (median 8 cm) in the malignant group and 2–16 cm (median 5·8 cm) in the benign group (Table 1). However, only four (10%) subjects with metastatic pheochromocytoma presented initially with primary tumours smaller than 5 cm compared to 33·3% subjects with benign pheochromocytoma ($P < 0·001$) (Table 1). Malignant adrenal pheochromocytomas were diagnosed more frequently at the right side compared to the benign tumours, but this difference did not reach statistical significance. Incidence of bilateral tumours was similar in both groups of subjects (Table 1).

The analysis of catecholamine levels showed that subjects with malignant pheochromocytoma secreted significantly more often norepinephrine, thereby having a so-called noradrenergic phenotype ($P = 0·04$) (Table 3). On the contrary, significantly increased epinephrine levels, or so-called adrenergic phenotype, were more common in subjects with benign pheochromocytomas, although not reaching statistical significance ($P = 0·06$) (Table 3). Isolated norepinephrine secretion was the most common type of catecholamine secretion in subjects with malignant pheochromocytoma. In the benign group, co-secretion of norepinephrine and epinephrine was the most frequent type of catecholamine secretion (Table 2). Only one patient with malignant pheochromocytoma (4 cm primary pheochromocytoma diameter) secreted only epinephrine, whereas isolated epinephrine secretion was found in 11 (10·1%) subjects with benign pheochromocytoma (Table 3). No significant difference was found in the frequency of dopamine secretion or in the number of subjects without catecholamine or metanephrine secretion, which are so-called biochemically silent tumours (Table 3).

Table 1 Baseline characteristics of subjects with metastatic and benign pheochromocytoma

Parameter	Metastatic pheochromocytoma (n = 41)	Benign pheochromocytoma (n = 108)	P
Gender (male/female)	19/22	44/62	NS
Age (years) (min; max)	41·4 ± 14·7 (6;83)	50·2 ± 13·7 (21;78)	< 0·001
Age < 40 years (%)	19 (46·3%)	26 (24·1%)	0·008
Tumor dimension (cm) (min; max)	8·38 ± 3·27 (2·4;17)	6·18 ± 2·75 (2;16)	< 0·001
Number of tumors ≥ 5cm	37 (90%)	72 (66·7%)	0·002
Right adrenal	28 (68·3%)	59 (54·6%)	0·11
Left adrenal	12 (29·3%)	44 (40·7%)	NS
Bilateral adrenal involvement	4 (9·8%)	5 (4·6%)	NS
Syndromic presentation	9 (25·7%)	11 (14·7%)	0·13
SDHB	2	0	
MEN 2	4	2	
VHL	3	5	
NF1	0	4	

SDHB, succinate dehydrogenase subunit B; MEN 2, multiple endocrine neoplasia type 2; VHL, von Hippel–Lindau; NF1, neurofibromatosis type 1; NS, nonsignificant.

Table 2 Histopathologic markers of potential malignancy in subjects with metastatic and benign pheochromocytoma

Parameter	Metastatic pheochromocytoma (n = 29)	Benign pheochromocytoma (n = 108)	P
Vascular invasion	5 (17·2%)	11 (10·2%)	NS
Capsular invasion	4 (13·8)	16 (16·7%)	NS
Increased mitotic figures	2 (6·9%)	8 (7·4)	NS
Necrosis	8 (27·6%)	0 (0%)	< 0·001
Invasion to adjacent adipose tissue	2 (6·9%)	5 (4·6%)	NS

NS, nonsignificant.

Table 3 Biochemical patterns of subjects with metastatic and benign pheochromocytoma

Parameter	Metastatic pheochromocytoma (n = 41)	Benign pheochromocytoma (n = 108)	P
Norepinephrine	15 (36·6%)	22 (20·4%)	0·04
Norepinephrine + epinephrine	13 (31·7%)	51 (47·7%)	0·06
Norepinephrine + epinephrine + dopamine	8 (19·5%)	12 (11·2%)	NS
Norepinephrine + dopamine	3 (7·3%)	5 (4·7%)	NS
Epinephrine	1 (2·4%)	11 (10·1%)	0·11
No catecholamine secretion	1 (2·4%)	6 (5·6%)	NS
Positive norepinephrine	39 (95·1%)	90 (83·3%)	0·046
Positive epinephrine	22 (53·6%)	74 (69·2%)	0·06
Positive dopamine or without catecholamine secretion	12 (29·3%)	23 (21·3%)	NS

Norepinephrine, epinephrine and dopamine denote elevated either catecholamines or its metabolites either in urine or in plasma.
NS, nonsignificant.

The median time from the diagnosis of the primary tumour to the diagnosis of metastatic spread was 3·6 years (in subjects younger 40 years, the median time was 7 years and in subjects older 40 years 3·1 years; P = 0·24) (Table 4; Fig. 1). In eleven subjects (27%), malignant disease was diagnosed either at the time of diagnosis of a primary tumour or during the first year after diagnosis of pheochromocytoma. In almost half of the subjects (18 patients), metastatic involvement was discovered later than 5 years after the diagnosis of primary tumour (the longest interval between the diagnosis of primary tumour and metastases was 24 years) (Table 4). Sixteen subjects older than 40 years (76%) developed metastases during the 5 years after diagnosis of pheochromocytoma compared to only seven subjects younger than 40 years (32%) (P = 0·02). No other differences were found between subjects younger and older than 40 years regarding tumour size and type catecholamine secretion. Four subjects with malignant pheochromocytoma had a range of 17 to 27 years between the diagnosis of primary tumour and metastases, but they were not included in the analysis because of the inability to obtain information regarding the dimension of the primary tumour. The most common sites of metastatic location were lymphatic nodes (66%), liver (49%), bones (49%) and less frequently lungs (34%) (Table 4).

Survival analysis subjects with metastatic pheochromocytoma (median time for the censored observations was 9·0 years, range from 0·6 to 27·3 years) revealed significantly longer survival of subjects secreting epinephrine (P = 0·02; Fig. 2) and shorter survival of subjects who produced dopamine or presented with biochemically silent tumours (P = 0·04; Fig. 2). No survival differences were found according to metastatic involvement (liver, lung and bones).

Table 4 Time to the development of distant metastases and metastatic locations in subjects with metastatic pheochromocytoma

Parameter	Metastatic pheochromocytoma (n = 41)
Time to diagnosis of malignancy (years)	3·6 (0·42; 9) (min 0; max 24)
Diagnosis of malignancy at presentation or until 1 year of diagnosis of pheochromocytoma	11 (26·8%)
Diagnosis of malignancy 1–5 years after diagnosis of pheochromocytoma	12 (29·3%)
Diagnosis of malignancy more than 5 years after diagnosis of pheochromocytoma	18 (43·9%)
Metastatic locations	
Lymphatic nodes	27 (65·9%)
Liver	20 (48·8%)
Bone	20 (48·8%)
Lung	14 (34·1%)
Mediastinum	4 (9·8%)

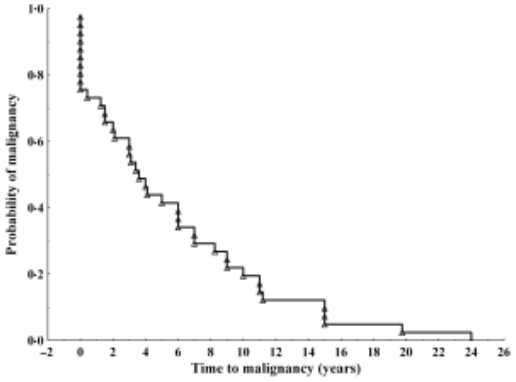

Figure 1 Distribution of time intervals from the diagnosis of primary tumour to diagnosis of metastases.

Discussion

In the present retrospective analysis that specifically focused on subjects with pheochromocytoma as the adrenal tumour, we have found that subjects with metastatic disease presented with larger primary tumours at significantly younger age and

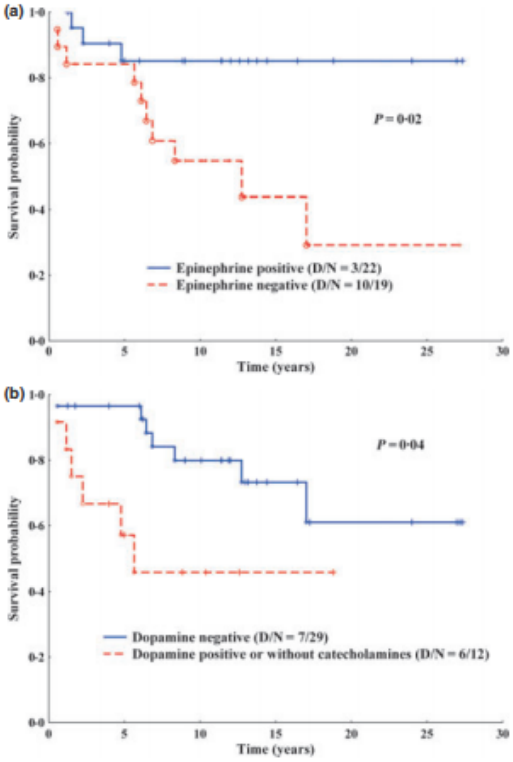

Figure 2 Survival of subjects with metastatic pheochromocytoma in respect to catecholamine secretion. Epinephrine or dopamine denotes either maternal catecholamines or its metabolites in urine or in plasma; D, number of deaths; *N*, total number of subjects.

produced more frequently norepinephrine compared to subjects with apparently benign tumours. There was no statistical difference in the gender distribution and in frequency of mutations of genes associated with the presence of pheochromocytoma.

Our findings that metastatic pheochromocytomas present with larger primary tumours than benign ones suggest that the size of a primary tumour is an important predictor of malignancy. Thus, as found in the present study, 90% of subjects with metastatic pheochromocytomas presented with primary tumours larger than 5 cm. These findings that a tumour dimension plays an important role as a risk factor in the development of metastatic spread are further supported by previous

reports [16,17]. In the study of Kimura *et al.*, [17] the dimension and weight of primary adrenal pheochromocytomas were significantly higher in subjects with malignant tumours compared to subjects with apparently benign disease. Similar results were found in the study of Shen *et al.* [16] where dimension of primary tumours was significantly larger in malignant pheochromocytomas compared to benign tumours. In the study of Amar *et al.*, [3] the smallest diameter of the primary tumour (extra-adrenal or adrenal origin) that developed metastases was 5 cm. In contrast, Linnoila *et al.* [19] showed that weight of primary tumours of malignant pheochromocytomas or paragangliomas was significantly higher than benign tumours, but no correlation was found between tumour weight and development of metastases. However, the number of tumours available for the analysis in this study was small and no attention was paid to discriminate between primary adrenal and extra-adrenal tumours. Similarly, Thompson [11] found no significant difference in the primary tumour dimension between malignant and benign adrenal pheochromocytoma.

In contrast, only 10% of subjects with metastatic pheochromocytomas presented with tumours smaller than 5 cm. These results do not support findings of the study of Gupta *et al.*, where 47% of subjects with metastatic pheochromocytoma presented with primary tumours < 5 cm [20]. The smallest tumour diameter in our study was 2·4 cm which is in agreement with other authors who also found primary tumours < 3 cm which presented in the later course with distant metastases [11,21].

Until now, many authors have attempted to find predictors of malignancy in pheochromocytoma but no scoring system has been proven reliable [10]. The strongest predictors are extra-adrenal locations and the presence of mutation of the *SDHB* gene. Although the mostly used PASS score (includes vascular, capsular or adipose tissue invasion, large nests or diffuse growth, focal or confluent necrosis, high cellularity, tumour cell spindling, cellular monotony, increased mitotic figures, atypical mitotic figures, profound nuclear pleomorphism and hyperchromasia) developed by Thompson [11] might be of some value [21,22], other authors reported about its limitations because of high interobserver and intraobserver variation [12]. According to our results, only necrosis occurred more frequently in malignant tumours but no difference was found in vascular and capsular invasion or invasion into adipose tissue and in the number of mitotic figures. Thus, present results of histopathologic findings support previous findings of Linnoila *et al.* and Kimura *et al.* [17,19] who concluded that necrosis but not capsular or vascular invasion is indeed an important risk factor for the development of metastatic pheochromocytoma.

Age

From the clinical point of view, age at time of a primary tumour associated with metastatic disease later on and time interval from the diagnosis of the primary tumour to the diagnosis of metastases are also important facts. First, pheochromocytomas associated with the development of metastatic disease were diagnosed at significantly younger age compared to subjects with benign pheochromocytoma, which is in the agreement with Kimura *et al.* [17]. Higher age in subjects with benign disease might be explained by the fact that the number of subjects with pheochromocytoma diagnosed incidentally during abdominal ultrasound, CT or MRI performed from other reasons is still increasing [23,24]. Second, the number of subjects who developed metastases at least 5 years after the diagnosis of primary tumour was also higher in younger subjects compared to older subjects with malignant pheochromocytoma. One explanation could be that younger subjects may not be so complaint as older ones to undergo a regular follow-up which is mandatory in all subjects with diagnosed pheochromocytoma [25]. The age-dependent function of immune system and behaviour of tumour cells may be additional important factors that could contribute to the explanation of present findings.

Biochemistry

Analysis of biochemical phenotypes revealed that norepinephrine secretion in subjects with metastatic pheochromocytoma was more frequent than those with benign tumours. On the contrary, there was a tendency to have more frequent epinephrine secretion in subjects with benign tumours. Epinephrine production is the latest step in the catecholamine synthesis catalysed by phenylethanolamine-N-methyltransferase. Thus, the failure of neuronal apoptosis during embryological development [26] in dopaminergic or noradrenergic chromaffin progenitors would be expected to occur earlier than in more fully mature adrenergic chromaffin cells and this may also explain younger age in subjects with malignant pheochromocytoma. Secretion of epinephrine or its metabolite metanephrine which may be regarded as a marker of cell differentiation was associated with better survival of subjects with metastatic PHEO whereas dopamine or its metabolite metanephrine was an indicator of a worse outcome of subjects with metastatic pheochromocytoma.

Both groups of subjects did not differ in the presence of tumours secretion either only dopamine or being biochemically silent. This could be explained by the relatively low number of subjects who have been tested positive for mutations in the *SDHB* gene which has been found to be strongly associated not only with malignant disease but also with either dopamine-secreting tumours or no catecholamine production at all [3,27,28].

Finally, it should be noted that a genetic background was not found to play any important role in the pathogenesis of malignant pheochromocytoma. However, only two patients with

mutation of the *SDHB*-gene, which is strongly associated with a malignant behaviour of extra-adrenal pheochromocytoma, were included in the present study. Therefore, larger samples of SDHB-related pheochromocytomas are needed to confirm these preliminary observations. This would most likely require multi-institutional studies because these tumours are quite rare.

Our study has several limitations. First, genetic testing could not be performed in all subjects and genetic testing for other pheochromocytoma susceptibility genes [*isocitrate dehydrogenase 1* and *-2*, *SDHAF2*, *TMEM127* and *SDHC* (29–31)] was not carried out. Second, the interval for follow-up in 49% of patients was < 5 years and these patients will need to be followed for a longer time period.

In conclusion, subjects with metastatic adrenal pheochromocytoma presented with larger primary tumours that secreted more frequently norepinephrine and were significantly younger at the time of diagnosis of primary tumour than subjects with benign adrenal pheochromocytoma. According to our data, a minimal tumour diameter of 5 cm might be considered an important risk factor for the metastatic spread in subjects with pheochromocytoma. Almost half of the subjects with metastatic pheochromocytoma developed metastases at least 5 years after the diagnosis of the primary tumour which underlines the importance of very close lifelong follow-up of subjects with pheochromocytoma/paraganglioma. We propose that the optimal interval for subjects at higher risk of malignancy (younger subjects with tumours larger than 5 cm and secreting norepinephrine) would be for biochemical testing every 6 months and for morphological testing (CT or MRI scanning) once a year. On the other hand, testing on yearly basis would be sufficient for subjects with lower risk of malignancy (older subjects with tumours smaller than 5 cm and secreting epinephrine).

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Disclosure

The authors have nothing to disclose.

Address

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9.2.4 Publikace 4

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Hereditární feochromocytom a paragangliom.

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V tomto souhrnném články jsme charakterizovali problematiku PHEO/PGL, včetně klinických projevů, genetického podkladu a vztahu k jednotlivým syndromům, je zde popsán algoritmus genetického vyšetřování pacientů. Z pohledu klinického pracovníka jsou zde popsány postupy vedoucí k odhalení přítomnosti PHEO/PGL (biochemické vyšetření, zobrazovací metody), doporučení k pooperačnímu sledování pacientů, preventivní sledování u zdravých nosičů patogenní mutace s vysokým rizikem PHEO/PGL a nutnost multidisciplinárního přístupu při péči o pacienty zahrnující spolupráci endokrinologů, pediatrů, chirurgů včetně urologů, otorinolaryngologů, cévních chirurgů nebo neurochirurgů, anesteziologů, klinických genetiků, klinických onkologů.

Hereditární feochromocytom a paragangliom

Hereditary pheochromocytoma and paraganglioma

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Souhrn

Feochromocytomy a paragangliomy jsou nádory vznikající z chromafinních buněk, mohou metabolizovat, skladovat, ale ne vždy vylučovat katecholaminy. Typickými projevy feochromocytomu nebo paragangliomu jsou hypertenze (trvalá i záchvatovitá), palpitace, bolesti hlavy a pocení. Se vznikem těchto nádorů je dnes spojeno deset genů a předpokládá se, že další budou objeveny. Oba typy nádorů se vyskytují také v rámci genetických syndromů: syndromu familiární paragangliomatózy (geny *SDH, SDHAF2*), syndromu von Hippel-Lindau (gen *VHL*), syndromu mnohočetné endokrinní neoplazie typu 2 (gen *RET*) a neurofibromatózy typu 1 (gen *NF1*). U některých syndromů jsou tyto nádory prvním a jediným manifestovaným onemocněním. Některé typy mutací, především v genu *SDHB*, jsou spojeny s vysokým počtem maligních onemocnění, která jsou v současné době standardními postupy nevyléčitelná. Z těchto důvodů je nezbytné provádět genetické vyšetření nejen u pacienta, ale v celé rodině, a nabídnout nositelům mutací dlouhodobé nebo celoživotní sledování a případně včasnou léčbu. Péče o pacienty s těmito onemocněními proto vyžaduje multidisciplinární spolupráci a měla by být prováděna pouze ve specializovaných centrech, která mají s tímto druhem onemocnění dostatečné zkušenosti.

Klíčová slova

feochromocytom – paragangliom – genetické vyšetření – sledování

Summary

Pheochromocytomas and paragangliomas are tumors arising from chromaffin cells. These tumors produce catecholamines and are typically found with symptoms and signs that may include hypertension (persistent or episodic), palpitations, headache and sweating. So far, 10 different genes have been associated with both tumors and other genes are expected to be detected. Pheochromocytoma and paraganglioma can occur as a part of genetic syndromes – familial paragangliomas (*SDH* genes, *SDHAF2* gene), von Hippel-Lindau syndrome (*VHL* gene), multiple endocrine neoplasia type 2 (*RET* gene), and neurofibromatosis type 1 (*NF1* gene). These tumors may be the first and only manifestation of these genetic syndromes. Patients with *SDHB* mutations are at high risk to develop malignant disease and unfortunately current therapeutic options for malignant form of disease are poor. Genetic testing plays a key role in the management of these tumors and therefore not only index patients with pheochromocytoma but also relatives should be tested. Management of this disease requires multidisciplinary cooperation and should be performed in the specialized medical centres.

Key words

pheochromocytoma – paraganglioma – genetic testing – follow up

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Zodpovědné geny: *SDHA, SDHB, SDHC, SDHD, SDHAF2 (SDH5), VHL, NF1, RET, MAX, TMEM 127*
Typ dědičnosti: autozomálně dominantní
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Charakteristika onemocnění

Feochromocytomy jsou relativně vzácné tumory vycházející z chromafinních buněk a produkující katecholaminy. Incidence feochromocytomu se odhaduje v rozmezí 0,5–1 diagnostikovaný případ na 100 000 obyvatel za rok. Klasické feochromocytomy se nachází v dřeni nadledvin, extraadrenální feochromocytomy, které nazýváme paragangliomy, se mohou nacházet v oblasti břicha, pánve, hrudníku a krku. Ačkoli tyto nádory vycházejí ze stejné tkáně, mají odlišné klinické projevy, genetický základ a prognózu. Feochromocytomy se objevují přibližně u 0,5 % pacientů s hypertenzí a u 4 % pacientů s adrenálním incidentalomem. V dnešní době je asi jedna třetina feochromocytomů diagnostikována jako náhodně zjištěný tumor nadledvin [1–4].

V odborné veřejnosti byly feochromocytomy známy jako nádory 10 %. Do roku 2000 bylo 10 % těchto nádorů považováno za dědičné v rámci genetických syndromů. Výsledky současných studií ukazují přítomnost kauzálních mutací u přibližně 30–40 % feochromocytomů nebo paragangliomů. Familiární formy jsou často multifokální nebo bilaterální a objevují se v mladším věku než sporadické případy [1,3,5,6].

Klinický obraz

Nejčastějšími příznaky feochromocytomu bývá hypertenze (80–90 % případů), která může být jak setrvalá, tak i záchvatovitá, palpitace (60 %), bolesti hlavy (50 %), bledost (40 %), pocení a psychické obtíže zahrnující úzkost a paniku (35 %). Typický je také váhový úbytek [7–10].

Malignita je u feochromocytomu a paragangliomu definována jen dle výskytu vzdálených metastáz (nejčastěji lymfatické uzliny, kosti, játra, plíce). Doba přežití u pacientů s metastatickým feochromocytomem a paragangliomem je velmi variabilní – někdy je průběh velmi fulminantní a v některých případech mohou pacienti přežívat i více než 20 let [8,9].

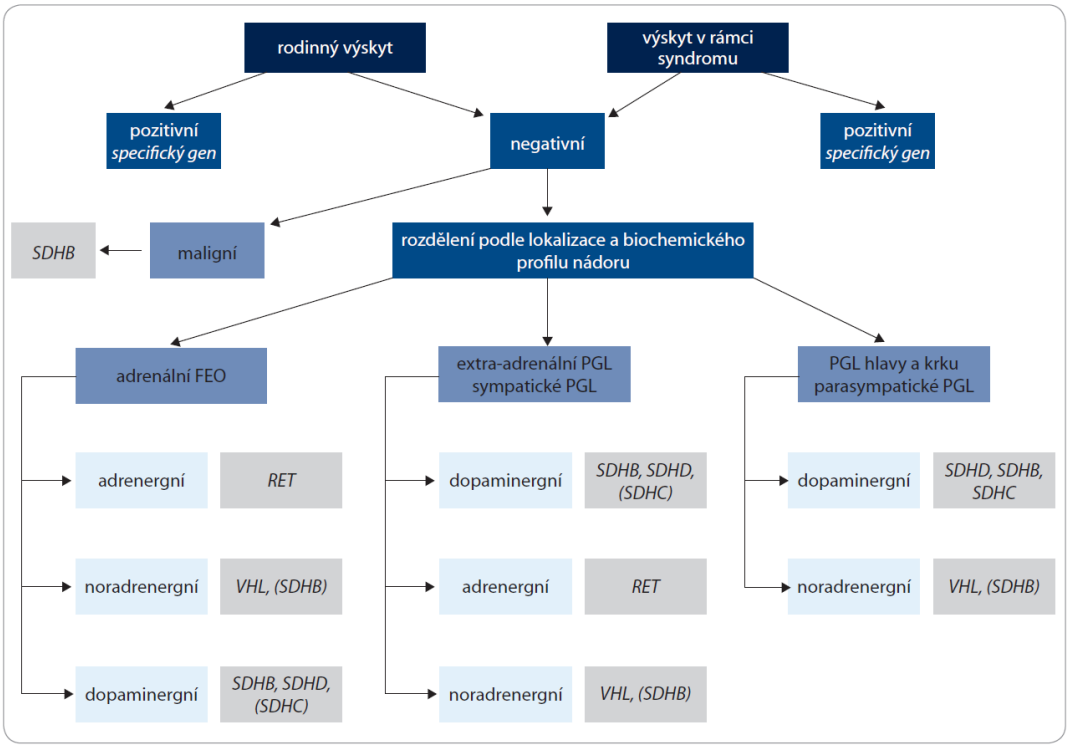
V dnešní době však není vzácností, že feochromocytom probíhá zcela asymptomaticky, na druhé straně jeho první manifestací může být i život ohrožující komplikace, jako je arytmie, infarkt myokardu, hypertenzní krize nebo cévní mozková příhoda. Feochromocytomy jsou geneticky velmi odlišné nádory. Jejich vznik je spojen s řadou dnes známých genů a předpokládá se objevení dalších, jak tomu bylo například v loňském roce u genu *MAX* [11]. Feochromocytomy mohou být také součástí genetických syndromů: syndromu mnohočetné endokrinní neoplazie 2. typu (zárodečné mutace v *RET* protoonkogenu), von Hippel-Lindauova syndromu (mutace v tumor supresorovém genu *VHL*) a také Recklinghausenovy neurofibromatózy (mutace genu *NF1*). V rámci těchto syndromů se jen vzácně vyskytují paragangliomy břicha nebo krku [1,6,12].

Další geny, které jsou příčinou především paragangliomů, kódují podjednotky mitochondriálního enzymu sukcinátdehydrogenázy (geny *SDHA, SDHB, SDHC, SDHD* a *SDHAF2*) a způsobují syndrom PGL1-5 [1,12]. Jednotlivé geny jsou vyšetřovány postupně podle klinického obrazu onemocnění (obr. 1). Jedná se o tumor supresorové geny a příčinou je ztráta heterozygozity. Ztráta wild type alely v tumoru společně se zárodečnou mutací znamená destabilizaci sukcinátdehydrogenázového komplexu a nefunkčnost enzymu [13,14]. Charakteristika jednotlivých genů podílejících se na vzniku feochromocytomu nebo paragangliomu je uvedena v tab. 1.

Sukcinátdehydrogenáza (též komplex II) je enzymatický komplex, který katalyzuje oxidaci sukcinátu na fumarát. Sukcinátdehydrogenáza se skládá ze čtyř různých podjednotek, SDHA je flavoprotein obsahující flavinadenindinukleotid, SDHB je FeS protein (obsahuje komplex železa a síry), SDHC a SDHD jsou hydrofobní proteiny kotvící komplex k membráně. Ačkoli jednotlivé podjednotky jsou součástí stejného proteinového komplexu, mutace v jednotlivých genech vedou k rozdílnému klinickému fenotypu [13].

Gen *SDHD*, syndrom paragangliomu typ 1 (PGL1); (OMIM 602690): mutace genu *SDHD* vedou k rozvoji onemocnění téměř výhradně, jsou-li zděděny od otce (parent-of-origin efekt) [15], vyskytují se především u krčních paragangliomů, méně často u paragangliomů hrudníku a břicha a feochromocytomů, metastatická forma onemocnění se vyskytuje zřídka [16]. Penetrance u nosičů *SDHD* mutace je vcelku vysoká a pohybuje se mezi 87 a 100 %. Průměrný věk pacientů s PGL1 syndromem se pohybuje mezi 20. a 40. rokem [13].

Gen *SDHFA2 (SDH5)*, syndrom paragangliomu typ 2 (PGL2); (OMIM 601650): gen *SDH5* kóduje protein,



Obr. 1. Algoritmus genetického vyšetření pacientů s feochromocytomem nebo paragangliomem.

který zajišťuje inkorporaci FAD kofaktoru SDHA podjednotky sukcinátdehydrogenázy, která je nezbytná pro správnou funkci SDH komplexu [16]. Mutace genu *SDHAF2* vykazují stejně jako změny v genu *SDHD* parent-of-origin efekt a vedou nejčastěji k multifokálním krčním paragangliomům v mladém věku [17]. Výskyt mutací není častý a genetické vyšetření se provádí, nejsou-li nalezeny změny v genech *SDHD, SDHC* nebo *SDHB* [13].

Gen *SDHC*, syndrom paragangliomu typ 3 (PGL3); (OMIM 602413): mutace genu *SDHC* vedou nejčastěji k rozvoji solitárních krčních paragangliomů, ale vzácně též byly zaznamenány případy sympatických paragangliomů a feo-

Tab. 1. Charakteristika onemocnění: DO = dopamin; E = epinefrin; NE = norepinefrin; NMN = normetanefrin; MN = metanefrin; MT = metoxytyramin.

Gen	Lokus	Riziko malignity	Primární výskyt	Biochemie
SDHA	5p15	neznámé	bez predilekce	neznámé
SDHB	1p 36,13	31–71 %	extraadrenálně	DO nebo MT, MN, NMN
SDHC	1q 21	nízké	paragangliomy hlavy a krku	MNM, MN, DO nebo MT, žádné
SDHD	11q 23	< 5 %	paragangliomy hlavy a krku (multifokální výskyt)	MNM, MN, DO nebo MT, žádné
SDHAF2 (SDH5)	11q 13,1	nízké	paragangliomy hlavy a krku (multifokální výskyt)	neznámé
VHL	3p 25–26	5 %	adrenálně	NMN, NE
NF1	17q 11,2	12 %	adrenálně	MN, MNM
RET	10q 11,2	< 5 %	adrenálně (bilaterálně)	E, MN
MAX	14q23,3	střední	adrenálně (bilaterálně)	neznámé
TMEM127	2q11,2	nízké	adrenálně	MN

chromocytomů. Paragangliomy vzniklé vlivem mutací genu *SDHC* jsou však daleko méně časté než *SDHB* nebo *SDHD* paragangliomy a vyskytují se v méně než 1 % případů. Většinou se jedná o benigní formu onemocnění. Popsán byl pouze jeden případ malignity. Změny v tomto genu jsou vzácné a genetické testování se provádí u *SDHD* a *SDHB* negativních pacientů [12,13].

Gen *SDHB*, syndrom paragangliomu typ 4 (PGL4); (OMIM 185470): tento syndrom se nejčastěji projevuje přítomností sympatických paragangliomů, méně často se vyskytují parasympatické krční paragangliomy. Na rozdíl od převážně benigních *SDHC* a *SDHD* tumorů, *SDHB* paraganliomy mohou velmi často metastazovat a vyskytují se v mladém věku. Nedávno publikovaná data prokázala, že u dětí s metastatickým paragangliomem se mutace *SDHB* genu vyskytovala až v 83 % případů [18]. Mutace genu *SDHB* mohou predisponovat nosiče k rozvoji dalších nádorových onemocnění jako např. Carnaey-Stratakisova syndromu, gastrointestinálních stromálních tumorů (GIST) [19,20], renálního karcinomu různých typů, neuroblastomu a papilárnímu karcinomu štítnice [21,22].

Carney-Stratakis syndrom (OMIM 606864) je nedávno popsaný syndrom, který zahrnuje výskyt multicentrického paragangliomu a multifokálního GISTu. U pacientů s tímto syndromem se nachází alelické ztráty chromozomálních lokusů pro geny *SDHB* nebo *SDHC*. Germinální mutace v genech *SDH* se nachází u 15 % pacientů s GIST bez rodinného výskytu paragangliomu [23]. Obdobně ve 14 % případů pacientů s renálním karcinomem byla prokázána přítomnost germinální mutace genu *SDHB*.

Gen *SDHA*, syndrom paragangliomu typ 5 (PGL5); (OMIM 600857): zárodečné mutace tohoto genu způsobují především neurodegenerativní onemocnění, tzv. Leighnův syndrom. Výskyt mutací u sporadických feochromocytomů a paragangliomů je vzácný a objevuje se méně než ve 3 % případů [16].

Gen *TMEM127*; (OMIM 613403): tento gen kóduje vysoce konzervovaný a široce exprimovaný transmembránový protein a nachází se na cytoplazmatické membráně i v cytoplazmě. Protein má tři transmembránové domény, ale nemá zatím žádnou známou funkční doménu a hraje roli v přenosu proteinů mezi cytoplazmatickou membránou, Golgiho komplexem a lyzozomy [24]. Mutace genu *TMEM127* vedou téměř výhradně k rozvoji feochromocytomů (často bilaterálních) [16], nicméně byly i zaznamenány případy extraadrenálních a krčních paragangliomů, zřídka jsou však maligní [25].

Gen *MAX* (MYC – associated factor X); (OMIM 154950): tento gen kóduje transkripční faktor, jako homodimer nemá transkripční doménu a působí inhibičně, naopak s c-myc onkogenem vytváří heterodimer, který se váže na DNA a je aktivační pro řadu buněčných pochodů. Zárodečné mutace v genu *MAX* byly nalezeny u 1 % pacientů s feochromocytomem, u nichž nebyla nalezena mutace v jiných genech [11].

Gen *VHL*, syndrom von Hippe-Lindau; (OMIM 608537): příčinou vzniku von Hippel-Lindauova syndromu jsou zárodečné mutace *VHL* tumor supresorového genu. Klinickými příznaky VHL syndromu je přítomnost hemangioblastomů retiny a CNS, světlobuněčný karcinom ledvin, feochromocytom, cysty pankreatu a ledvin, tumory saccus endolymphaticus a papilární cystadenomy. Zárodečné mutace *VHL* vykazují autozomálně dominantní charakter dědičnosti. U 90 % nosičů se vyvine onemocnění do 60 let věku, 3–5 % pacientů se sporadickým feochromocytomem může mít zárodečnou mutaci ve *VHL* genu. Feochromocytomy asociované s VHL syndromem jsou nejčastěji adrenální a bilaterální, mohou se objevit i extraadrenálně. V rámci VHL syndromu bylo nalezeno přibližně 5 % maligních feochromocytomů [2,4,26].

Gen *RET*, syndrom mnohočetné endokrinní neoplazie 2. typu (MEN-2); (OMIM 171400): syndrom mnohočetné endokrinní neoplazie je podmíněn mutacemi v *RET* protoonkogenu. Klinickými projevy tohoto syndromu jsou medulární karcinom štítné žlázy, primární hyperparatyreóza (MEN 2A) a další znaky jako např. ganglioneuromy a marfanoidní habitus (MEN 2B). Medulární karcinom štítné žlázy mají téměř všichni pacienti MEN-2 syndromu a většinou předchází diagnóze feochromocytomu vyskytující se s penetrancí více než 90 % do 50 let věku. Feochromocytom se objevuje přibližně u 50 % pacientů s MEN-2. Protoonkogen *RET* kóduje transmembránový tyrosinkinázový receptor [4]. Mutace v tomto genu se nejčastěji nachází v exonu 10 a 11 u MEN 2A a v exonu 16 u MEN 2B. Paragangliom se v rámci syndromu MEN 2 vyskytuje zcela vzácně. Feochromocytomy se nachází uni- i bilaterálně [2].

Gen *NF1*, neurofibromatóza – typ 1 (NF-1); (OMIM 162200): neurofibromatóza – typ 1 (nazývaná též *morbus von Recklinghausen* či periferní typ neurofibromatózy). Jde o tumor-supresorový gen, jehož produkt (neurofibromin) je součástí intracelulární signální kaskády spojené s RAS-kinázou. Jedná se o relativně časté AD dědičné onemocnění (1 : 2 500–4 000 novorozenců). Projevuje se abnormálním růstem podpůrných buněk centrální a periferní nervové soustavy (gliomy, Schwannovy buňky aj.) s výraznou predispozicí ke vzniku benigních i maligních nádorů. Klinický průkaz neurofibromatózy 1. typu je v současné době považován u feochromocytomu za dostatečně průkazný pro zařazení pacienta do skupiny feochromocytomů asociovaných s neurofibromatózou [4].

Mezi klinické projevy neurofibromatózy patří tzv. *„café-au-lait spots"* (skvrny barvy „bílé kávy", v 90 % se objeví do pěti let věku), neurofibromy (mnohočetné tumorózní uzlíky; kutánní, subkutánní a plexiformní; hlavně v axilách a tříslech), Lischovy uzlíky (hamartomy duhovky), NF 1 je asociován s větším počtem různých neuroendokrinních tumorů včetně feochromocytomu, který ovšem není příliš častý a vyskytuje se přibližně v 0,1–5,5 % případů [2].

Biochemické vyšetření

Jak již bylo zmíněno, feochromocytomy nebo paragangliomy produkují, ale ne vždy vylučují, katecholaminy. Naopak jejich metabolity metanefriny bývají vylučovány prakticky vždy, a vykazují tedy lepší senzitivitu než mateřské katecholaminy. Dle posledních prací vykazují nejlepších výsledků plazmatické metanefriny s ohledem na lepší specificitu ve srovnání s močovými metanefriny [27].

Z tohoto důvodu je pravděpodobně nejvýhodnější používat stanovení plazmatických metanefrinů. Odpadá tak horší spolupráce pacientů při sběrech moči. Pro volbu způsobu stanovení metanefrinů je však nejdůležitější zkušenost pracoviště s danou metodou. Některé tumory (především ty méně diferencované, jako jsou paragangliomy na podkladě genu *SDHB* nebo paragangliomy hlavy a krku) mohou produkovat pouze dopamin nebo methoxytyramin. I přes výrazné zlepšení biochemické diagnostiky však u některých tumorů (typicky paragangliomy hlavy a krku a pak i nádorů na podkladě mutace genu *SDHB*) nemusíme prokázat jakékoli zvýšení metanefrinů a tyto nádory považujeme za sekrečně němé. Zde nám může pomoci i stanovení jiného markeru neuroendokrinních nádorů – chromograninu, který může být jediným zvýšeným nálezem u pacientů s jinak sekrečně němým paragangliomem na podkladě mutace genu *SDHB* [7–10,28].

Zobrazovací metody

K lokalizaci tumorů by se mělo přistoupit až po předchozím potvrzení biochemickými metodami, pokud nebyl nádor zjištěn dříve. Základním diagnostickým nástrojem pro nádory v oblasti břicha je v našich podmínkách CT. MR použijeme jedině při alergii na kontrastní látku nebo u dětských pacientů (pro snížení radiační zátěže). Naopak pro oblast hlavy a krku je vhodné použití MR. Pro potvrzení diagnózy (CT i MR nejsou specifické) a také k vyloučení mnohočetného (a metastatického) postižení využíváme metod nukleární medicíny – pro feochromocytomy scintigrafii s [^{123}I]- metajodobenzylguanidinem a pro paragangliomy hlavy a krku s [^{111}In]-octreotidem. V dnešní době se ale dostávají do popředí metody založené na positronové emisní tomografii – jednak [^{18}F]-fluorodopa (ta je nejvýhodnější pro paragangliomy hlavy a krku) a pro pacienty s mutací genu *SDHB* nebo metastatickým postižením [^{18}F]-fluorodeoxyglukóza [8–10,28].

Pooperační sledování

Všichni pacienti by měli být po operaci sledováni. Zvláštní pozornost zaslouží pacienti s největším rizikem možné recidivy nebo vzniku metastáz. Na prvním místě to jsou pacienti s mutací genu *SDHB* [14,29,30]. U nich je vhodné pravidelné pooperační sledování s maximálním intervalem šesti měsíců s biochemickým (stanovení metanefrinů) a případně i morfologickým (funkčním) vyšetřením. Pokud by se však jednalo o nádor sekrečně němý, tak v tomto případě je nutné se spolehnout jen na morfologické (funkční) vyšetření. Mezi rizikové z hlediska vzniku metastáz řadíme dále pacienty s funkčním paragangliomem a s objemným feochromocytomem, naopak příznivý průběh můžeme očekávat u starších nemocných s malými feochromocytomy, které produkují adrenalin [30]. Vyšší pravděpodobnost recidivy můžeme očekávat i u pacientů s feochromocytomy vzniklými na podkladě mutace genů *VHL*, *RET* či *NF1*, u nichž je vysoká pravděpodobnost bilaterálního postižení. I zde je vhodné pravidelné, nejlépe šestiměsíční sledování. Toto vyšetřovací schéma volíme zpočátku i u ostatních pacientů s feochromocytomy těsně po operaci s možností prodloužení na roční interval v dalším průběhu sledování. Pacienty s paragangliomem hlavy a krku bychom měli sledovat i po operaci, především ty s mutací *SDHD* genu, neboť je zde velké riziko vzniku dalšího nádoru. Pro všechny pacienty s těmito nádory doporučujeme sledování ve specializovaných centrech s dostatečnou zkušeností v léčbě těchto nádorů.

Preventivní sledování u zdravých nosičů patogenní mutace s vysokým rizikem feochromocytomu a paragangliomů

1. Klinické vyšetření specialistou včetně kontroly krevního tlaku a případně další rutinní vyšetření, jako je stanovení krevního obrazu a základní biochemie jednou ročně.
2. Zobrazovací metody: sonografie krku, břicha po dvou letech; magnetická rezonance hlavy a krku, hrudníku, břicha a malé pánve (v závislosti na druhu mutace, u *SDHB* nosičů zvážit celotělové vyšetření) po dvou letech; u pozitivního nálezu potvrzení PET/CT vyšetřením. Metody lze v ročním intervalu střídat.
3. Plazmatické, případně močové metanefriny včetně metoxytyraminu (metoxytyramin vždy u *SDHC* nosičů) jednou ročně. Pokud byl jediným zvýšeným laboratorním ukazatelem u probanda chromogranin, tak i stanovení chromograninu (chromogranin je nutný u *SDHB* nosičů).

Návrhy preventivní péče pro osoby s von Hipple-Lindau syndromem, NF1 syndromem a MEN2 syndromem byly publikovány v Klinické onkologii 2009, Suppl 22. Péče o pacienty s feochromocytomem a paragangliomem nebo o rizikové osoby vyžaduje multidisciplinární přístup (endokrinolog, pediatr, chirurg včetně urologa, otorinolaryngolog, cévní chirurg nebo neurochirurg, anesteziolog, klinický genetik, klinický onkolog a další). Při nízké incidenci, obtížné předoperační přípravě a možnosti maligního onemocnění je nepochybně s výhodou pro pacienta centralizace těchto pacientů včetně genetického vyšetření.

Preventivní péče o děti ve vysokém riziku by měla být prováděna ve FN Motol a FN Brno. Preventivní péče o dospělé by měla probíhat v centrech, která mají dostatečnou zkušenost s diagnostikou a léčbou těchto nádorů.

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9.2.5 Publikace 5

Vicha A, **Musil Z,** Pacak K.

Genetics of pheochromocytoma and paraganglioma syndromes: new advances and future treatment options.

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Souhrn obsahu:

V tomto článku jsme popsali rozdělení PHEO/PGL do skupin (clusterů) podle výsledků transkriptomových studií. V první skupině se nachazí nádory u kterých se vyskytují mutace v genech *VHL, SDHx (SDHA, SDHB, SDHC, SDHD a SDHAF2)*, u druhé skupiny tumorů se nachází změny v genech *RET, NF1, KIF1Bβ, TMEM 127* a *MAX*.

Cluster 1

Mutace SDHx/VHL způsobují nesprávnou degradaci a akumulaci HIF-1/HIF-2α což vede např. k pseudohypoxii, zvýšené angiogenesi a vyššímu výskytu reaktivních forem kyslíku. HIF-1/HIF-2α tvoří heterodimer a působí jako transkripční faktor. Sukcinát, který se díky nefunkčnosti SDH komplexu akumuluje v mitochondriální matrix posléze proniká do cytosolu a inhibuje aktivitu prolyl hydroxylázy. Hydroxylovaný HIF-α je rozpoznáván VHL proteinem a posléze degradován v proteasomu. Je-li gen *VHL* mutován, protein se netvoří, HIF-α nemůže být degradován a proto dochází k jeho hromadění.

Cluster 2

Tato skupina nádorů je spojena s aktivací kinázových signálních drah, které se podílí na celé řadě buněčných procesů. Mutace *RET* jsou spojovány se zvýšenou aktivací PI3K/v-Akt signální dráhy. Gen *NF1* kóduje GTPázu aktivující protein neurofibromin, který se účastní přenosu signálu v RAS a mTOR signálních drahách. Proto mutace *RET* a *NF1* vedou k aktivaci PI3K/AKT/mTOR a RAS/RAF/MAPK signálních drah. Mutace *TMEM127* zvyšují aktivitu mTOR nezávisle na *RET* a *NF1*, zatímco mutace genu *MAX* vedou k nesprávné regulaci MYC-MAX-MXD1 dráhy, která je spřažena s drahou mTOR.

Jelikož je zatím nejúčinnějším způsobem léčby chirurgické odstranění, pochopení specifických genetických mechanismů, které vedou k rozvoji PHEO/PGL dává do budoucna naději na výběh a použití správné cílené terapie

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Genetics of pheochromocytoma and paraganglioma syndromes: new advances and future treatment options

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Abstract

Purpose of review—To summarize the recent advances in the genetics of pheochromocytoma and paraganglioma (PHEO/PGL), focusing on the new susceptibility genes and dividing PHEOs/PGLs into two groups based on their transcription profile.

Recent findings—Recently, *TMEM127*, MYC-associated factor X, and hypoxia-inducible factor (HIF) 2α have been described in the pathogenesis of PHEOs/PGLs. Thus, now about 30–40% of these tumors are linked to the germline mutations, which also include mutations in the *VHL*, *RET*, *NF1*, *SDHx*, and *SDHAF2* genes. Furthermore, PHEOs/PGLs have been divided into two groups, cluster 1 (SDHx/VHL) and cluster 2 (RET/NF1), based on the transcription profile revealed by genome-wide expression microarray analysis.

Summary—PHEOs/PGLs are the most inherited tumors among (neuro)endocrine tumors. Future approaches in genetics, including whole-genome sequencing, will allow the discovery of additional PHEO/PGL susceptibility genes. The current division of PHEOs/PGLs into cluster 1 and 2 provides us with additional knowledge related to the pathogenesis of these tumors, including the introduction of new treatment options for patients with metastatic PHEOs/PGLs. New discoveries related to the role of the *HIF-1*/*HIF-2*α genes in the pathogenesis of almost all inherited PHEOs/PGLs may call for a new regrouping of these tumors and discoveries of new treatment targets.

Keywords

gene; mutation; paraganglioma; pheochromocytoma; succinate dehydrogenase

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Conflicts of interest

There are no conflicts of interest.

INTRODUCTION

Pheochromocytomas and paragangliomas (PHEOs/PGLs) are rare neuroendocrine tumors that produce catecholamines [1]. PHEOs/PGLs arise from three distinct parts of the neural crest: the adrenal medulla (PHEOs) and the sympathetic and parasympathetic paraganglia (PGLs) [2]. Initially, the pioneering work of Neumann *et al*. [3] showed that about one-quarter of these tumors were hereditary. According to the recent publications, up to 30–40% of these tumors are genetically inherited [3,4,5■■,6]. Now, the group of susceptibility genes includes the following genes: the von Hippel–Lindau (*VHL*) tumor suppressor gene, the rearranged during transfection (*RET*) protooncogene, the neurofibromatosis type 1 (*NF1*) tumor suppressor gene, genes encoding the four subunits (A, B, C, and D) of the succinate dehydrogenase (*SDH*) complex, and a gene encoding the enzyme responsible for flavination of the SDHA subunit (*SDHAF2*). In addition to these PHEO/PGL susceptibility genes, two other genes, KIF1Bβ and PHD2, have also been associated with PHEO/PGL development, although very rarely. Recently, three other genes, *TMEM127*, MYC-associated factor X (*MAX*), and hypoxia-inducible factor 2α (*HIF2A*) (both somatic and germline mutations found), have been described [7■,8,9■■,10,11,12■]. Somatic mutations in the known PHEO/PGL susceptibility genes have been reported as an extremely rare event, but recently, Burnichon *et al*. [13] detected somatic mutations of the RET and VHL genes in about 14% of sporadic PHEOs/PGLs. Also, Welander *et al*. [14] suggest that the NF1 gene constitutes the most frequent (24%) target of somatic mutations so far known in sporadic PHEOs. Furthermore, somatic mutations in *MAX* were found in 1.65% of tumors [15]. Thus, the proportion of all patients with PHEOs/PGLs because of the gene mutations described above is estimated to be approximately 50% at present (Table 1). In the present article, we focused on the role of new genes linked to the pathogenesis of PHEOs/PGLs and on clustering hereditary PHEOs/PGLs based on their transcriptional profile as described previously [13]. Discoveries of new genes and specific transcriptional profiles for PHEOs/PGLs will further provide new treatment strategies for these tumors.

TMEM127 MUTATION

Recently, Qin *et al*. [8] reported heterozygous germline mutations in the *TMEM127* gene in seven patients affected by PHEO. The *TMEM127* gene, located on chromosome 2q11, encodes a transmembrane protein of 238 amino acids. TMEM127 is a highly conserved and broadly expressed protein with three transmembrane regions, but has no known functional domains. The TMEM127 protein associates dynamically with the endosomes and may participate in the protein trafficking between the plasma membrane, the Golgi, and lysosomes [8]. Tumors with *TMEM127* mutations have a transcription signature comparable to that of *RET*-mutated and *NF1*-mutated PHEOs. However, neither RAS activation nor AKT phosphorylation was seen, indicating that TMEM127 loss is not identical to either NF1 or RET [8]. *In vitro* and in primary tumors, it was found that TMEM127 functions as a negative regulator of the mammalian target of rapamycin (mTOR), or more specifically of mTORC1, but the mechanisms underlying this interaction have not yet been established. However, *TMEM127* knockdown, leading to an increase in the phosphorylation of mTORC1 targets, results in larger cells with higher rates of proliferation and hyperphosphorylation of mTOR effector proteins [8]. Thus, *TMEM127* is a new tumor-suppressor gene involved in

hereditary PHEOs/PGLs. *TMEM127* missense, frame shift, and nonsense mutations were detected in all three coding exons of the gene, but no large *TMEM127* deletions or duplications were found. Genetic studies of PHEO/PGL patients indicate a low prevalence of *TMEM127* mutations (~2% of all cases negative for other PHEO/PGL susceptibility mutations) [8,17■,18]. A unique finding in these patients was the older average age (42 years) at presentation, which is similar to sporadic cases, but older than that of carriers of mutations in other susceptibility genes [8,17■,18]. In most cases, *TMEM127* mutation carriers suffered from PHEOs only (unilateral as well as bilateral tumors) and secreted a high level of metanephrines. Only two mutations have been reported in a few patients with PGLs [19], one associated with multiple PHEO and retroperitoneal PGL and the other with bilateral carotid PGLs [8,17■,18,20]. Malignancy has been reported rarely [8,17■,18].

MAX MUTATION

In 2011, Comino-Mendez *et al*. [7■] identified *MAX* as a new PHEO tumor-suppressor gene in three independent patients with familial antecedents of the disease. The protein encoded by the MAX gene is a member of the basic helix–loop–helix leucine zipper (bHLHZ) family of transcription factors. The MAX protein is a ubiquitous, constitutively expressed protein that plays a central role in controlling the MYC/MAX/MXD1 (MAX dimerization protein 1) axis. MYC activates transcription binding to E-box DNA recognition sequences in target gene promoters through heterodimerization with MAX; heterodimers of MAX with MXD1 antagonize MYC-dependent cell transformation by transcriptional repression of the same E-box target DNA sequences [21,22]. *MAX* mutations are associated with bilateral PHEOs and an apparent paternal transmission of the disease [7■]. In a large international study, Burnichon *et al*. [15] confirmed that *MAX* germline mutations are responsible for PHEOs and PGLs in 1.12% of cases. In this study, patients with pathogenic *MAX* mutations had adrenal tumors, including 13 with bilateral or multiple PHEOs within the same adrenal gland. *MAX*-related thoracoabdominal PGLs were also found in the same study [15]. Thirty-seven percent of these patients had familial antecedents. Malignant disease developed in about 10.5% of patients [15]. However, Comino-Mendez *et al*. [7■] found metastasis at diagnosis in three out of eight probands. Therefore, mutations of *MAX* can be associated with a high risk of malignancy. Furthermore, somatic mutations in *MAX* were found in 1.65% of tumors [15]. The mutations were found in all five exons of the MAX gene, but were especially frequent in exons 3 and 4, corresponding to some of the most important residues within the conserved bHLH–Zip domain of MAX. The majority of the mutations led to the presence of truncated proteins, which resulted in the absence of the protein, as determined by immunohistochemistry [15]. How *MAX* mutations contribute to the pathogenesis of PHEO/PGL remains unclear. However, the ability of MYC to function independently of MAX has been demonstrated [23]. Therefore, Cascón and Robledo [21] suggested that the pivotal role of MAX in the MYC/MAX/MXD1 network is related more to the repression rather than to the activation of the MYC network.

HIF2A MUTATION

Zhuang *et al*. [9■■] identified novel somatic mutations in the gene encoding HIF-2α in multiple PGLs and duodenal somatostatinomas associated with polycythemia, suggesting

the existence of a new syndrome. Also, Favier *et al.* [24] found somatic HIF2A mutations that may lead to the pathogenesis of PHEO in a female patient. The existence of a new syndrome (potentially to be named Pacak–Zhuang syndrome) was then confirmed in a larger series of female patients [16■]. These patients were found to have polycythemia either at birth or in childhood together with multiple PGLs and somatostatinomas. Subsequently, Lorenzo *et al.* [12■] reported a novel *HIF2A* germline mutation in a patient with congenital polycythemia with multiple PGLs.

HIFs are transcription factors controlling energy, iron metabolism, erythropoiesis, development, glycolysis, and other cell functions [25,26■]. The HIF-β subunit is constitutively expressed, whereas the α subunits are inducible by hypoxia [27]. When these proteins are dysregulated, they often contribute to tumorigenesis and cancer progression [28,29]. However, mutations in the genes encoding HIFs have not previously been identified in any cancer. Dominantly inherited gain-of-function mutations of *HIF2A* were previously found to be associated with an increase of erythropoietin and congenital polycythemia [30,31]. In HIF-2α-related polycythemia with or without PGLs, these mutations have been found mainly at hot spots in exon 12 [9■■,30,31]. HIF-2α gene mutations were found to disrupt prolyl hydroxylation of HIF-2α and, in turn, recognition by the VHL protein, resulting in a failure of HIF-2α ubiquitylation and its degradation. Thus, the longer half-life of the mutant HIF-2α protein resulted in the upregulation of downstream HIF-2α targets (EDN1, EPO, GLUT1, and VEGF), which is currently believed to be the pathogenic mechanism that leads to tumor development [9■■]. Very recently, we have also identified a somatic HIF2A gene mutation in a patient with multiple PHEOs associated with polycythemia (unpublished observation).

THE CLUSTERS OF HEREDITARY PHEOCHROMOCYTOMAS AND PARAGANGLIOMAS BASED ON TRANSCRIPTIONAL PROFILE

Hereditary and sporadic PHEOs/PGLs can be divided into two groups based on the transcription profile revealed by the genome-wide expression microarray analysis. The first group (cluster 1) includes tumors carrying *VHL* and *SDHx* (*SDHD*, *SDHB*, *SDHC*, *SDHA*, and *SDHAF2*) mutations and also accounts for about 30% of sporadic tumors [13,32,33]. The second group (cluster 2) represents the tumors carrying *NF1*, *RET*, and *KIF1B*β mutations, and also includes about 70% of sporadic tumors [5■■,13,34■,35■]. The new *TMEM127* and *MAX* genes are most likely associated with cluster 2 and *HIF2A* with cluster 1 [9■■,12■,13,15].

Cluster 1

VHL/SDHx mutations lead to impaired degradation and accumulation of HIF-1/HIF-2α and display signatures of pseudohypoxia, angiogenesis, increased reactive oxygen species (ROS), and reduced oxidative response, resulting in changes in the cell metabolism (energy metabolism regulation). HIF-α heterodimerizes with HIF-β and acts as an active transcription factor. Succinate that accumulates in the mitochondrial matrix owing to SDH dysfunction leaks out into the cytosol, where it inhibits the activity of HIF-1/HIF-2α prolyl hydroxylase enzymes (PHDs – PHD1, PHD2, and PHD3, also known as EglN2, EglN1, and

EglN3, respectively) that hydroxylate two prolyl residues [36]. Hydroxylated HIF-α is recognized by the VHL protein and marked for degradation in the proteasome. If the *VHL* gene is mutated, the protein is not formed, so HIF-α cannot be degraded and therefore accumulates. Burnichon *et al*. [13] showed that *SDHx*-related and *VHL*-related PHEOs/PGLs shared overexpression of several genes involved in angiogenesis and the hypoxic pathway. Some genes were specifically overexpressed in *SDHx*-related tumors. These genes are involved in transcription regulation, protein transport, proliferation, energy metabolism, and cell adhesion. Also, several specifically overexpressed genes have been found in *VHL*-related tumors. These genes are *EGLN3* and *KISS1R*, as well as genes involved in glycolysis [13]. Burnichon *et al*. [13] found, which was also confirmed by Lopez-Jimenez *et al*. [33], that although *VHL*/*SDHx*-related tumors are associated with pseudo-hypoxia, some HIF target genes were differentially expressed between *SDHx* and *VHL*-related tumors. Most of these genes, such as *ENO1*, *BNIP3*, or *CA9*, are considered to be HIF-1α-specific targets and were specifically induced in *VHL*-related tumors [13]. Therefore, these tumors were further divided into cluster 1A (*SDHx*) and cluster 1B (*VHL*).

The *SDHB* mutation is associated with malignancy and poor prognosis [37]. Burnichon *et al*. [13] found genes specifically overexpressed in *SDHB*-related PHEOs/PGLs, including MMP24, DSP, SIX1, LGR5, LAPTM4B, and β-catenin, most of which are important in the development of metastasis.

Cluster 2

Cluster-2-related PHEOs/PGLs are linked together by the activation of kinase signaling pathways driven by the oncogenes that involve translation, initiation, and protein synthesis, and genes involved in neural/neuroendocrine identity [13,32, 35■,38–40]. The proto-oncogene RET is a tyrosine kinase receptor primarily expressed in the neural crest cells. *RET* mutations have been associated with increased activation of PI3K/v-Akt signals [41]. *NF1* encodes for the protein neurofibromin, a GTPase-activating protein in the RAS signaling cascade and mTOR signaling pathway [42,43]. Thus, *RET* and *NF1* mutations lead to activation of the PI3K/AKT/ mTOR and RAS/RAF/MAPK signaling pathways. *TMEM127* mutations enhance mTOR activity independent of the RET and NF1 pathways. Finally, MAX gene mutations result in the dysregulation of the MYC–MAX–MXD1 network connected with mTOR pathway function [15].

FUTURE TREATMENT

Whereas the most optimal treatment for benign PHEOs/PGLs is surgical resection, therapy for malignant/metastatic disease is unsatisfying at best. Understanding the specific genetic alterations of various PHEOs/PGLs will undoubtedly open promising new options for targeted therapies in the near future. Also, clustering PHEOs/PGLs by expression alterations can lead us to choose certain treatment targets. Thus, expression microarrays can be a more powerful tool for the detection of target genes and associated pathways involved in PHEOs/PGLs in the future [13,15,24,28,42,43].

Yang *et al*. [44■] have recently demonstrated that the loss of SDHB function was because of a reduction in the mutant protein half-life by rapid proteasome degradation. The authors

found that histone deacetylase inhibitors (HDACi) stabilized the half-life of mutated SDHB proteins and their activity. Direct activation of PHD by the activator KRH102053 increases HIF-1/HIF-2α hydroxylation and promotes its degradation [45,46].

Metastatic SDHx-related PHEO/PGL overexpresses heat shock protein 90 (HSP90), a molecular chaperone that assists in binding to HIF-1/HIF-2α and promotes its stability by preventing ubiquitination and proteasomal degradation of HIF-1/HIF-2α [25,47,48]. Thus, the inhibitors of HSP90, such as geldanamycin and analogs 17-allylaminogeldanamycin (17-AAG; tanespimycin) and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG; alvespimycin) or other new ones, are promising novel anticancer therapeutic agents [49,50]. Small-molecule inhibitors of glucose transporter 1 (GLUT1), such as WZB117 or STF-31, downregulate glycolysis and inhibit cancer cell growth *in vitro* and *in vivo* [51,52]. Favier *et al*. [53] showed that cluster 1 tumors display angiogenic markers such as vascular epithelial growth factor (VEGF), its receptors, HIF-2α, angiopoietin-2, and the endothelin receptors ETA and ETB. Thus, these results suggest that there is a rationale for antiangiogenic therapy, including targeting the VEGF pathway using either humanized monoclonal anti-VEGF antibodies (Bevacizumab) or small tyrosine kinase inhibitors such as sunitinib or sorafenib [53]. Also, targeting HIF-1/HIF-2α by HIF-1α inhibitors (direct inhibitor PX-478 and indirect inhibitor PX-12) has shown antitumoral activity inhumantumor xenografts in mice and also seems to be promising for malignant PHEO/PGL [54–56].

Favier *et al*. [53] showed that the mTOR pathway was potentially activated in half of PHEOs/PGLs. Nolting *et al*. [57] showed that the combination treatment with dual NVP-BEZ235 (PI3K/mTORC1 inhibitor) and lovastatin (inhibitor of ERK signaling) had a significant additive effect in mouse PHEO cells and resulted in inhibition of both AKT and mTORC1/p70S6K signaling without ERK upregulation.

The new therapeutic options outlined above are expected to be introduced in the near future.

CONCLUSION

In the near future, next-generation sequencing technology is predicted to replace conventional sequencing methods and a stepwise procedure for genetic screening will likely no longer be required. Similarly, whole-genome sequencing will allow the discovery of newer PHEO/PGL susceptibility genes. Molecular targeted therapies will appear as the most promising strategies for the management of patients with metastatic PHEO/PGL.

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Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 248).

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KEY POINTS

- *TMEM127*, *MAX*, and *HIF2A* are the new susceptibility genes for PHEOs/PGLs.
- New syndromes (such as Carney–Stratakis syndrome, comprised PGL and GIST, and a new syndrome potentially to be named Pacak–Zhuang syndrome, comprised multiple PGLs and duodenal somatostatinomas associated with polycythemia in women) have been introduced.
- Genome-wide studies led to the identification of somatic mutations in about 14% of sporadic PHEOs/PGLs.
- Hereditary and sporadic PHEOs/PGLs are currently divided into two groups (SDHx/VHL and RET/NF1) based on the transcription profile revealed by genome-wide expression microarray analysis or by other approaches.
- Deep knowledge of the genetic changes and expression profiles will introduce new targeted therapy for malignant PHEOs/PGLs.

Table 1

Genotype-phenotype correlations in pheochromocytoma/paraganglioma because of mutations in susceptibility genes

Gene	Locus	Syndrome	Inh.	Malignant PHEO/PGL	Single PHEO	Bilateral PHEO	TAPGL	HNPGL	Multiple PGLs	Biochemical phenotype
SDHA	5p15		AD	?	–	–	+	+	–	?
SDHB	1p 36.13	PGL4	AD	+++	++	+	+++	++	++	MT, NMN, MTY or NS
SDHC	1q 21	PGL3	AD	±	±	–	+	++	+	MT, NMN or NS
SDHD	11q 23	PGL1	AD P1	+	+	+	++	+++	+++	MT, NMN, MTY or NS
SDHAF2	11q 13.1	PGL2	AD P1	?	–	–	–	+++	++	?
VHL	3p25-p26	VHL	AD	+	++	+++	+	±	+	NMN
NF1	17q11.2	NF1	AD	+	+	±	–	–	–	MN, NMM
RET	10q11.2	MEN	AD	±	++	++	–	–	–	MN, NMM
MAX	14q23.3		AD P1	+	++	++	–	–	–	Mixed: NMN + MN
TMEM127	2q11.2		AD	±	+++	++	±	±	±	MN
HIF2A	2p21-p16		Somatic[a]	?	±	?[b]	++	–	++	NMN

AD, autosomal dominant; HN/PGL, head and neck paraganglioma; Inh, inheritance; MN, metanephrine; MTY, methoxytyramine; NMN, normetanephrine; NS, nonsecreting; PGL, paraganglioma; PHEO, pheochromocytoma; PI, paternal inheritance; TAPGL, thoracic or abdominal paraganglioma; ?, unknown. *SDHx*-related paragangliomas can be also associated with SDH-deficient gastrointestinal stromal tumors (GISTs). This autosomal-dominant familial paraganglioma and GIST syndrome is known as Carney–Stratakis syndrome [11]. Recently, Pacak–Zhuang syndrome including paraganglioma, somatostatinoma, and polycythemia in women has been introduced. This syndrome is associated with somatic HIF2A gain-of-function mutation [16■].

[a] Rarely germline.

[b] Taieb, Pacak, unpublished observation.

9.2.6 Publikace 6

Vosecka T., Vicha A., Zelinka T., Jencova P., Pacak K., Duskova J., Benes J., Guha A., Stanek L., Kohoutova M., Musil Z.

Absence of BRAF mutation in pheochromocytoma and paraganglioma.

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Shrnutí obsahu:

V tomto článku jsme se zaměřili na detekci mutací v genu *BRAF* a případném použití inhibitorů u pacientů s PHEO/PGL. Tento gen kóduje protein BRAF, který se v buňce účastní přenosu signálu a regulaci MAP/ERK signální dráhy. Tato signální dráha se podílí na buněčném dělení, diferenciaci a sekreci.

Frekvence *BRAF* mutací v nádorech se liší. Vyšší výskyt je pozorován u melanomů, vlasatobuněčné leukémie a histiocytóze z Langerhansových buněk, zatímco např. u neuroblastomů, kolorektálního karcinomu, non-Hodgkinova lymfomu a astrocytomů je výskyt nižší.

Celkem jsme vyšetřili 64 vzorků s PHEO/PGL (32 mužů a 32 žen, věkové rozpětí 7 až 77 let). Charakteristika souboru a použité metody jsou detailně popsány v článku.

V námi sledovaném souboru jsme prozatím nenašli žádnou změnu v genu *BRAF*. Tento výsledek naznačuje, že mutace tohoto genu je vzácná.

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Absence of BRAF mutation in pheochromocytoma and paraganglioma

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Pheochromocytomas and Paragangliomas (PHEO/PARA) are rare endocrine tumors originating from the adrenal medulla. More than 20 genes are involved in the tumorigenesis of these tumors, but a substantial part of the causative genetic events remains unexplained. A recent study has reported the presence of the activating *BRAF* V600E mutation in PCC, suggesting a role for BRAF activation in tumor development. Other studies have not find this mutation. This study investigates the occurrence of the *BRAF* V600E mutation in these tumors.

A cohort of 64 PHEO/PARA were screened for the *BRAF* V600E mutation using direct Sanger sequencing and QRT-PCR.

All cases investigated displayed wild-type without V600E BRAF mutation

Taken together with all previously screened tumors up to date, only 1 V600E *BRAF* mutation has been found among 427 PCCs. These findings imply that the V600E *BRAF* mutation is a rare event in PHEO/PARA.

Key words: pheochromocytoma, paraganglioma, BRAF, mutation

B-Raf is a protein produced by the human gene *BRAF* [1, 2]. B-Raf is a 766-amino acid protein composed of three conserved domains characteristics of the Raf kinase family. In active conformation, B-Raf forms dimers via electrostatic interactions of its kinase domains and hydrogen-bonding [3]. Cell growth is directed by signalling from the B-Raf protein, since it is a member of the Raf kinase family of growth signal transduction protein kinases. Hence, it plays a role in regulating the MAP kinase/ERKs signalling pathway, which in turn affects cell division, differentiation, and secretion. Human cancers have been associated with more than 30 mutations of the *BRAF*. In 90% of these cases, thymine is substituted with adenine at nucleotide 1799 (p. V600E) [4].

The frequency of *BRAF* mutations varies widely in human cancers. Higher frequencies are found in melanomas and nevi, hairy cell leukaemia, and Langerhans cell histiocytosis while rarely in other tumors such as non-Hodgkins lymphoma, colorectal cancer, astrocytoma, papillary thyroid carcinoma, non-small-cell lung carcinoma, lung adenocarcinoma, and neuroblastoma [5-13]. Furthermore, a high frequency of *BRAF* V600E mutations have been detected in ameloblastoma, a locally infiltrative odontogenic benign neoplasm [14].

To date, only one study has identified a *BRAF* mutation with an incidence of 1,2% (1/85) in pheochromocytoma and paraganglioma (PHEO/PGL) [15]. Paulson et al, summarized data from other studies and did not found V600E *BRAF* mutation in 0,3% (1/336) PHEO/PGL tumors [16, 17].

Similary to neuroblastoma which have incidence of *BRAF* mutation about 1%, PHEO is also tumor of the adrenal gland that arises from chromaffin cells located in the adrenal medulla. PGL arise from extra-adrenal chromaffin cells located in sympathetic (abdomen, pelvis) or parasympathetic (head and neck) ganglions [18, 19]. These tumors may produce and

secrete catecholamines and metanephrines [20-22]. Currently, there are about 20 known genes associated with PHEO/PGL pathogenesis [18, 23-25].

Here, we aimed to assess the presence of the *BRAF* mutation on a large population of PHEOs/PGLs and to further contribute to controversial view whether this mutation may or may not occur in these tumors [18, 26]. Since *BRAF* mutation is a very good treatment target, the presence of this mutation in some of these tumors could result in the use of B Raf inhibitors of metastatic forms for which there has not been effective treatment so far.

Materials and methods

Our study included 64 patients with PHEO/PGLs (32 men and 32 women, range from 7 to 77 years). Patient's samples were collected from the 3rd Department of Medicine, 1st Faculty of Medicine, Faculty Hospital and Department of Pediatric Hematology and Oncology, 2nd Faculty of Medicine, Prague, Czech Republic. The informed consent was obtained from all involved patients. Clinical characteristics of study objects are described in Table 1.

Table 1. Clinical and germline mutations characterization

Num.	Age (y)	sex	PHEO/ PARA	site	Volum (ml)	Metastases	Familiar	Germline mutation of SDHB, SDHD, RET, VHL genes
1	36	F	PHEO	Left	39	No	No	Neg.
2	16	M	PHEO	Right	n.d.	No	No	Neg.
3	12	F	PHEO	Bilateral	n.d.	No	No	VHL: c.602T>C, p.Leu201Pro
4	65	F	PHEO	Right	45	no	No	Neg.
5	45	F	PARA	Retroperitoneum	31	no	No	Neg.
6	13	F	PARA	Retroperitoneum	n.d.	no	No	SDHB: c. 589 – 600 dup. AGC ACC AGC TGC, p. Cys 196 dup. Ser 197 Thr 198 Ser 199 Cys 201
7	68	M	PHEO	Right	70	no	No	Neg.
8	35	F	PHEO	Left	120	no	No	Neg.
9	62	F	PHEO	Right	70	no	No	Neg.
10	7	M	PARA	Mediastinum	24	no	No	VHL: c.376G>A, p.Asp126Asn
11	64	F	PHEO	Left	42	no	No	Neg.
12	14	M	PHEO	Right	n.d.	no	No	Neg.
13	22	F	PHEO	Left	46	no	No	Neg.
14	35	M	PHEO	Left	80	no	No	Neg.
15	31	F	PHEO	Bilateral	10;30	no	No	RET: c.1901G>C, p.Cys634Ser; c.1921G>T, p.Ala641Ser
16	52	M	PHEO	Bilateral	6; hyperplasia	no	No	Neg.
17	59	F	PHEO	Left	63	no	No	Neg.
18	68	M	PHEO	Left	28	no	No	Neg.
19	60	M	PHEO	Right	13	no	No	Neg.
20	15	M	PHEO	Right	n.d.	no	No	Neg.
21	73	F	PHEO	Right	35	no	No	Neg.
22	31	F	PARA	Middle ear	n.d.	no	No	Neg.
23	27	F	PARA	Retroperitoneum	60	no	No	Neg.
24	41	M	PHEO	Left	100	no	No	n.d.
25	76	M	PHEO	Right	60	no	No	Neg.
26	23	M	PARA	Mediastinum	60	bone; GC; neck	Yes	Neg.
27	47	M	PHEO	Left	58	no	No	Neg.
28	13	M	PARA	Rertoperitoneum	86+80	no	No	Neg.
29	26	M	PARA	Retroperitoneum	35	no	No	SDHD: c.361C>T, p.Gln121X
30	65	F	PHEO	Right	50	no	No	Neg.
31	40	M	PHEO	Bilateral	30;14	no	No	Neg.
32	40	M	PHEO	Right	70	no	No	Neg.
33	68	F	PHEO	Right	80	no	No	Neg.
34	67	F	PHEO	Right	60	no	No	Neg.
35	77	F	PHEO	Right	65	no	No	Neg.
36	26	F	PHEO	Left	50	no	No	Neg.
37	20	M	PHEO	Bilateral	70;40	no	No	VHL: c.340+2T>C
38	34	M	PHEO	Right	35	no	No	Neg.
39	27	M	PARA	Neck	n.d.	no	No	SDHD: c.2T>A, p.Met1Lys

Table 1. Clinical and germline mutations characterization (continued)

Num.	Age (y)	sex	PHEO/ PARA	site	Volum (ml)	Metastases	Familiar	Germline mutation of SDHB, SDHD, RET, VHL genes
40	65	F	PHEO	Left	17	no	No	Neg.
41	15	F	PHEO	Left	n.d.	no	No	Neg.
42	24	M	PHEO	Bilateral	30;6,9	no	No	VHL: c.374A>C, p.His125Pro
43	47	M	PHEO	Right	55	no	No	Neg.
44	60	F	PHEO	Left	665	no	No	Neg.
45	42	M	PHEO	Right	12	liver; bone;lymph nodes; lung	No	Neg.
46	75	F	PARA	Pelvis	50	no	No	Neg.
47	64	F	PHEO	Left	38	no	No	Neg.
48	60	F	PHEO	Bilateral	30;hyperplasia	no	No	Neg.
49	61	M	PHEO	Left	95	no	No	Neg.
50	33	F	PHEO	Right	55	no	No	Neg.
51	57	F	PHEO	Left	60	no	No	VHL: c.351G>A, p.Trp117Ter
52	21	M	PHEO	Bilateral	60;25	no	No	Neg.
53	50	M	PHEO	Right	8	no	No	Neg.
54	28	F	PHEO	Left	55	no	No	Neg.
55	61	F	PHEO	Right	60	no	No	Neg.
56	51	M	PHEO	Right	50	no	No	Neg.
57	9	F		Retroperitoneum	360	no	No	Neg.
58	68	M	PHEO	Left	40	no	No	Neg.
59	14	F		Retroperitoneum	n.d.	no	No	n.d.
60	77	M	PHEO	Left	90	no	No	Neg.
61	66	M	PHEO	Right	55	no	No	Neg.
62	74	F		Zuckerkandel	27	no	No	Neg.
63	59	M	PHEO	Left	80	no	No	n.d.
64	59	F	PHEO	Left	110	no	No	Neg.

Table1. characterized clinical data and germline mutation status of SDHB, SDHD, VHL and RET genes.
PHEO/PARA-Pheochromocytoma/Paraganglioma; neg.- negative; n.d.- not done, M-male; F- female; GC-glomus caroticum

Genomic DNA was extracted from fresh or frozen peripheral blood using QIAamp DNA Mini Kit (Qiagene, USA). Somatic DNA was extracted from frozen tumour's samples after histological confirmation of PHEO/PGL. DNA was extracted by Puregene Core kit A (Qiagene, USA). Quality of DNA was checked by NanoDrop™ 2000/2000c Spectrophotometers (ThermoScientific)

Sanger sequencing. PCR primers for *BRAF* gene have been designed based on GenBank sequences using the Primer 3 software including intron-exon boundaries, reverse primer 5´- CTGTTCAAACTGATGGGACCC– 3´, forward primer 5´– TGCTTGCTCTGATAGGAAAATG– 3´. The BRAF PCR conditions are as follows 25 µl reaction mixture contained 1x PCR buffer (Fermentas), between 50-300 ng of genomic DNA as template, 1.5 mM MgCl2 (Fermentas), 25 pmol of each primer, 200 µM of each deoxynucleotide triphosphate (Fermentas), and 1.0 unit of TaqDNA polymerase (MBI Fermentas). Amplification conditions were included an initial denaturation at 94C° for 3 min., followed by 35 cycles of 45 sec at 94 C°, 45 sec at 60 C°, 1 min. at 72C° and final extension step running 5 min. at 72 C°. DNA fragments were sequenced in both directions using an automatic fluorescent ABI PrismTM 3130 Genetic Analyzer (PE Applied Biosystems) according to the manufacturer´s instructions.

QRT-PCR for detection of V600E *BRAF* mutation. PCR primers and probes (accession No. NG_007873), PCR conditions and results classification were designed by Lang et al. [27]. These primers and probes are targeted against each mutation, and a mutation-unspecific region was used as a reference amplicon. All unlabelled primers were synthesized by EastPort Praha, Czech Republic; and probes (TaqMan) were purchased from Applied Biosystems, Foster City, CA. Real-Time PCR Reference PCR was performed in a 25 µl reaction volume with HotstarTaq DNA polymerase, Qiagene, 900 nmol/L of each BRAF mutation-un-specific primer, 100 nmol/L of the BRAF probe, 112.5 nmol/L of each internal control primer, 25 nmol/L of internal control probe, and 5 µl of DNA of varying concentration. Allele-specific PCRs were performed according to the same protocol but using a concentration of 450 nmol/L of allele-specific primer. All real-time PCRs were performed on a system (TaqMan 7300 PCR System Applied Biosystems, Foster City, CA) under the following thermocycling conditions: 95°C for 10 minutes,

followed by 50 cycles of 90°C for 15 seconds and 60°C for 1 minute. Cycle threshold (Ct) values were recorded for reference PCR and for each allele-specific PCR, and corresponding Δ Ct values (ie, allele-specific Ct minus reference Ct) were calculated.

Results

Somatic activating *BRAF* mutations in exon 15 were investigated in 64 tumor samples. Initially, we used Sanger sequencing. Results for V600E *BRAF* mutation were validated by QRT-PCR. All of these samples were negative for *BRAF* mutation in exon 15.

Discussion

In the present study, which included 66 PHEOs/PGL, we did not confirm the presence of any *BRAF* mutations. This contrasts Luchetti et al., who detected V600E *BRAF* mutation in 1,2% (1/85) of these tumors. Until now, 427 PHEOs/PGLs were investigated for the presence of a *BRAF* mutation, which was only found] in 1 of these tumors, suggesting that the *BRAF* V600E mutation is a extremely rare genetic event in PHEO/PGL and would not serve as a target for new treatment options in metastatic PHEO/PGL.

Previous studies have demonstrated that tumor oncogene activation such as *RET*, *HIF2A*, and *HRAS* in PHEO/PGL may result in tumorigenesis of these tumors [15, 24, 26, 28, 29]. Furthermore, additional gene expression studies suggested that most PHEO/PGLs can be classified into two distinct groups (cluster1 and cluster 2) by transcription profiling: cluster 1 includes tumors that harbour mutations in genes linked to pseudohypoxia (*VHL, HIF2A, SDHA, SDHB, SDHC*, and *SDHD*) and cluster 2 contains tumors harbouring mutations in genes that are involved in the kinase signalling characterized by the activation of the PIK3/AKT/mTOR and RAS/RAF/ERK pathways *(RET, NF1, TMEM127, MAX,* and *HRAS*), both converging on the HIF-signaling pathway [30]. The proto-oncogene *RET* is a tyrosine kinase receptor primarily expressed in the neural crest cells. *RET* mutations have been associated with increased activation of PI3K/v-Akt signals. *NF1* encodes for the neurofibromin protein, a GTPase- activating protein in the RAS signaling cascade and mTOR signaling pathway. Thus, *RET* and *NF1* mutations lead to activation of the PI3K/AKT/mTOR and RAS/RAF/MAPK signaling pathways [24]. Thus, RAS/RAF/MAPK signaling pathways genes are a promising aim of mutations in PHEO/PGL. That supposition was confirmed by Luchetti et al. and other which found somatic *HRAS* mutation in PHEO/PGL [26, 15] very recently Luchetti et al. found V600E *BRAF* mutation in 1,2% (1/85 cases). We investigated 64 cases without any detection of the V600E *BRAF* mutation.

In conclusion, our results along with previous results, suggest that the BRAF V600E mutation is an extremely rare genetic event in PHEO/PGL.

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10. Diskuze:

Předkládaná postgraduální práce se zabývá relativně vzácnými, ale zrádnými nádory – PHEO/PGL. Jedná se o neuroendokrinní nádory, pro jejichž diagnostiku, léčbu a následné sledování je třeba spolupráce lékařů z různých oborů. Prvotním cílem bylo zavedení genetického vyšetření pro pacienty s PHEO/PGL a stanovení frekvence genetických změn, jsou dávány do souvislosti s rozvojem těchto druhů nádorů. Genetické vyšetření je důležité nejen pro samotné pacienty s PHEO/PGL, ale i pro jejich zatím bezpříznakové příbuzné. V našem souboru 108 pacientů jsme pomocí metody next generation sequencing (NGS) nalezli 25 mutací v 9 různých genech (23% záchyt), které jsou vyšetřovány v souvislosti s PHEO/PGL. Fishbein L. et al. 2017 detekovali 46 mutací ve sledovaném souboru 173 pacientů s PHEO/PGL (27% záchyt). Rovněž procentuální zastoupení mutací v jednotlivých genech našeho souboru (*SDHB* 24%, *RET* 20%, *SDHC* 16%, *SDHD* 4%, *TMEM* 127 8%, *VHL* 8%, *MAX* 8%, *MET* 8%, *NF1* 4%) se liší od jiných studií Dahia P.L. et al. 2014, Favier J. et al. 2015, Fishbein L. et al. 2017. Výsledky naší analýzy se tedy odlišují od výsledků některých doposud publikovaných studií, což může být vysvětleno složením a národnostní specifitou sledovaného souboru.

V další fázi jsme se snažili identifikovat klinické prediktory, které by mohly svědčit pro maligní průběh onemocnění. Analýza byla provedena u souboru 41 pacientů s metastatickým PHEO a kontrolního souboru 108 pacientů s benigním PHEO. Jak ukazují výsledky nejen naší studie (Zelinka T. et al. 2011), Kimura et al. 2005 pro metastatický průběh onemocnění svědčí výskyt primárního nádoru v mladším věku. Benigní formy PHEO jsou nalézány ve vyšším věku jako incidentalomy při ultrasonografii břicha, CT nebo MRI (Noshiro et al. 2000, Amar et al. 2005). Navíc počet pacientů, u kterých se objevily metastázy v průběhu pěti let od diagnozy primárního nádoru byl vyšší u mladších pacientů. U pacientů s metastatickou formou onemocnění byl častěji sekretován norepinefrin, na rozdíl od benigních nádorů, které častěji produkovaly epinefrin. Tento jev může být způsoben faktem, že epinefrin je syntetizován jako poslední krok při syntéze katecholaminů, a proto se dá očekávat selhání apoptózy během vývoje v dopaminergních nebo noradrenergních chromafinních progenitorech dříve než u zralých adrenergních chromafinních buněk (Lee et al. 2005). Z dalších výsledků vyplývá, že velikost primárního nádorů je dalším důležitým prognostickým faktorem, který může značit maligní průběh onemocnění 90 % pacientů s metastatickým PHEO mělo primární nádor větší než 5 cm. Námi nalezené výsledky potvrzují předchozí studie (Shen W.T. et al. 2004, Kimura N. et al. 2005), ve kterých velikost a hmotnost primárních nádorů u maligních pacientů byly významně větší než u benigních. Ve studii provedené Amar et al. 2007 nejmenší rozměr primárních

extraadrenálních nebo adrenálních tumorů byl 5 cm. Genetický profil pacientů s maligní formou se v naší studii neukázal jako významný, což může být způsobeno skutečností, že pouze dva pacienti měli mutaci v genu *SDHB*, který je jinak asociován s maligním průběhem onemocnění.

Léčba PHEO/PGL spočívá v odstranění nádoru, efektivní kontrole růstu tumorů a dalších přidružených symptomů. Díky neustále narůstajícím znalostem v oblasti buněčné signalizace, metabolismu, transkriptomu atd. mohou být jednotlivé molekulární podtypy cíleně léčeny. Antiangiogenní terapie může být použita u pacientů s mutacemi genů *SDH* či *VHL* (klastr 1 PHEO/PGL). Pro tento způsob léčby jsou vhodné monoklonální protilátky proti vascular endothelial growth factor (VEGF) jako např. Bevacizumab, nebo tyrosin kinázové inhibitory sunitinib či sorafenib (Tuthill M. et al. 2009, Joshua, A.M. et al. 2009, Hahn, N.M. et al. 2009, Jimenez, C. et al. 2009, Nemoto, K. et al. 2012). Zvýšená aktivace hypoxické signalizace je jednou ze známek charakteristických pro klastr 1 PHEO/PGL. Z tohoto důvodu by mohly být nadějné některé nové preparáty proti hypoxia inducible factor (HIF) (Welsh S.J. et al. 2003, Welsh, S. 2004, Chen W. et al. 2016. Nedávno byla s jistým úspěchem na myším modelu použita antracyklinová cytostatika, jako např. daunorubicin, doxorubicin a epirubicin, při léčbě metastatických forem PHEO/PGL. Inhibovala jak HIF-1, tak HIF 2α a díky těmto účinkům by mohla být použitelná u metastatických PHEO/PGL zvláště, mají-li změny v HIF signální dráze (Pang Y. et al. 2017).

Zvýšená Ras/Raf/Erk nebo PI3K/Akt/mTOR kinázová aktivita je pozorována u PHEO/PGL pacientů s mutacemi v genech *RET*, *NF1*, *TMEM127* a *MAX* (Attie T. et al. 1995, Ma X.M. et al. 2009, Burnichon N. et al. 2011). Zkoušky s inhibitorem mTOR (Everolimus), který byl podán pacientům s progresivním PHEO, přinesly rozporuplné výsledky (Druce, M.R. et al. 2009, Oh D.Y. et al. 2012). Mutace v genech *SDH*x vedou k akumulaci sukcinátu, což může způsobovat hypermetylaci DNA a histonů (Letouze E. et al. 2013, Killian J.K. et al. 2013), proto se některé demetylační léky (např. Decitabin) pro tento typ léčby testují (Letouze E. et al. 2013). Nesprávná funkce SDH komplexu je spojená i s poruchou NAD^+/NADH metabolismu u nádorových buněk a možné léčbě pomocí inhibitorů poly(ADP ribóza) polymerázy (PARP) (Sulkowski P.L. et al. 2018, Pang Y. et al. 2018). Dalším z možných léků používaných k léčbě PHEO/PGL mohou být inhibitory histon deacetylázy (HDAC). Tyto inhibitory navozují zástavu buněčného cyklu a apoptózu aktivací Notch1 signální dráhy nebo inhibicí nuclear factor erythroid 2-related factor2/ heme oxygenase 1 dráhy Nrf2/HO – 1 (Adler J.T. et al. 2008, Cayo M.A. et al. 2009, Li Z.Y. et al. 2016, Zhang Z. et al. 2017). Studie (Yang, C. et al. 2012) poukazují, že inhibitory HDAC zvyšují stabilitu SDHB proteinu a tím i podporují funkci mitochondriálního komplexu II.

Cílem našeho dalšího zájmu se stal gen *BRAF*, který kóduje serin/threoninovou kinázu BRAF. Tato kináza patří do RAF kinázové rodiny, která se prostřednictvím MAP/ERK signální dráhy účastní regulace buněčného růstu, sekrece a proliferace (An Y.H. et al. 2008). Počet mutací genu *BRAF* se liší u různých nádorů, vyšší frekvence se objevuje např. u melanomů a vlasatobuněčné leukémii, zatímco u kolorektálního karcinomu, neuroblastomů, plicních adenokarcinomů a astrocytomů se objevuje méně často (Shukla N. et al. 2012, Ahmadzadeh A. et al. 2014, Johnson D.B. et al. 2015, Sorbye H. et al. 2015). Počet mutací genu *BRAF* u pacientů s PHEO/PGL však podle naší i dalších studií není vysoký a nachází se přibližne u 1,2% nádorů (Vosecka T. et al. 2017, Luchetti A. et al. 2015, Paulsson J.O. et al. 2015). Použití inhibitorů BRAF tedy nemá zatím širšího využití.

11. Závěr:

Naše závěry podporují výsledky ostatních studií (Fishbein L et al. 2013, Brito JP et al. 2015) a doporučují provádění rutinních genetických vyšetření u všech pacientů s PHEO/PGL z několika důvodů:

a) až 40 % pacientů jsou nosiči germinálních mutací

b) pozitivita genetického vyšetření vede k zpřesnění diagnostických postupů

c) časnější záchyt eventuálního onemocnění u příbuzných jedinců probanda

d) mutace určitých genů mohou způsobovat další syndromické komorbidity nebo predisponovat k maligní formě onemocnění (geny *SDHB*, *FH*)

Díky rychlému rozvoji molekulárně biologických metod a použití techniky next generation sequencing (NGS) počet genů, které je možno rutinně analyzovat stále narůstá (Pillai S et al. 2016). V současné době testujeme 34 genů s výhledově rozšířením spektra analyzovaných genů.

12. Publikační činnost:

12.1 Publikace v časopisech s IF, které jsou součástí práce

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14. Přílohy:

14.1 Seznam obrázků a tabulek

Obrázky:

Tabulky:

14.2 Identifikační záznam

MUSIL, Zdeněk. *Molekulárně biologická analýza feochromocytomu a paragangliomu. [Molecular biological analysis of pheochromocytoma and paraganglioma]*. Praha, 2019. 100 s., 2 příl. Disertační práce. Univerzita Karlova, 1. lékařská fakulta, Ústav biologie a lékařské genetiky 1.LF UK a VFN. Školitelka: RNDr. Kateřina Hirschfeldová, Ph.D.